Mediators In A Murine Model For The Multiple Organ Dysfunction Syndrome

Mediators In A Murine Model For The Multiple Organ Dysfunction Syndrome

een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. Dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 19 april 2005
des namiddags om 1.30 uur precies
door Thomas Johannes Hermanus Volman
geboren op 14 juni 1974
te Born

Promotores:	Prof. Dr. R.J.A. Goris
	Prof. Dr. J.W.M. van der Meer
Co-Promotor:	Dr. T. Hendriks
Manuscriptcommissie:	Prof. Dr. J.G. van der Hoeven
	Prof. Dr. J. Schalkwijk
	Prof. Dr. H. Redl (Ludwig Boltzmann Institut für Experimentelle und Klinische Traumatologie, Wenen, Oostenrijk)
Paranimfen:	Ir. M.P.J. van der Gaag
	Ir. M.W.J. van Passel

Printing of this thesis was financially supported by:
AstraZenica B.V.
Hope Farms / ab diets, Woerden
Genzyme Europe B.V.

ISBN: 90-9019149-6
Cover design: Arjan Terwiel
Printed by: Print Partners Ipskamp

CONTENTS

1

Introduction and aim of the study

THE MULTIPLE ORGAN DYSFUNCTION SYNDROME

Care of critically ill patients has progressed significantly since world war two. An evolving ability to support vital organ system function during a period of otherwise lethal physiologic insufficiency has changed the process of hospital care. The development of techniques of hemodialysis, positive pressure ventilation and cardiovascular support have transformed life-threatening illness from a rapidly progressing lethal event to a chronic state that appears potentially survivable. Advances in the support of vital organ function have led to the institution of intensive care units and spawned an entirely new specialty, that of intensive care medicine.

Unfortunately, our increased ability to keep patients alive has yielded a new phenomenon: the sequential failure of organ systems that usually occurs after a lag period of days to weeks after various physiologic insults, which may include pancreatitis, trauma, burns, shock, severe infection, aspiration, multiple blood transfusions, and pulmonary contusions [1,2]. Previously known as "Multiple Organ Failure" (MOF), the name of this phenomenon has evolved to "Multiple Organ Dysfunction Syndrome" (MODS), because "dysfunction" is thought to be a better description of the continuum of changes that occur in organ systems. MODS is defined as the presence of altered organ function in a severely ill patient so that homeostasis cannot be maintained without intervention [3]. Regardless of the cause, MODS typically consists of the sequential dysfunction of several organ systems, beginning with the lungs and followed by hepatic, intestinal, renal, hematological and eventually cardial dysfunction, although the exact order may vary because of preexisting disease or the nature of the precipitating insult [4 5]. If fitting to specific definitions, the respiratory dysfunction is referred to as the acute respiratory distress syndrome (ARDS) [6]. Lung dysfunction may already be found within hours after the insult, and

consists of tachypnea and respiratory alkalosis, followed by a decrease in arterial pO_2.

Despite the astounding scientific progress in our understanding of infection, sepsis, trauma, cell and organ injury, shock, acute renal failure and acute respiratory failure, MODS remains the principal cause of death in surgical intensive care units. It is responsible for 50 to 80% of all surgical intensive care unit deaths and costs exceed $150.000 per patient [4]. Certain factors increase the probability for patients to develop MODS after an initial insult. These risk factors include inadequate or delayed resuscitation, a persistent infectious or inflammatory focus, baseline organ dysfunction (e.g. renal insufficiency), an age of >65 years, alcohol abuse, bowel infarction, malnutrition, diabetes, cancer, the presence of hematoma and the use of steroids [7]. Once MODS develops, the number of dysfunctioning organ systems is a crucial prognostic factor. Single-organ dysfunction has a mortality of 30 to 40%, whereas two- and three-organ dysfunction have mortalities exceeding 60 and 90%, respectively [7].

PATHOPHYSIOLOGY OF MODS

There are two clinical observations that strongly indicate that MODS is a systemic process. First, the organs where damage occurs are not necessarily directly injured or involved in the primary disease process. Second, there is usually a lag phase of days to weeks between the primary insult and the development of remote organ dysfunction. These observations suggest that endogenous circulating factors are involved in the pathophysiology of MODS. It is now generally believed that MODS is the result of a generalized inflammatory response. Inflammation is a rapid, highly amplified controlled humoral and cellular response to injury. The complement, kinin, coagulation and fibrinolytic cascades are triggered simultaneously with the activation of phagocytes and endothelial cells. This essentially local response is effective as

long as the inflammatory process is regulated appropriately to keep cells and mediators sequestered. There are four major events in the inflammatory process: vasodilatation, increased micro-vascular permeability, cellular activation/adhesion and coagulation. Vasodilatation and increased micro-vascular permeability at the site of injury increase locally available oxygen and nutrients and produce heat, swelling and tissue oedema. The local hemodynamic changes cause the five classical symptoms associated with local inflammation: rubor (erythema), tumor (edema), calor (heat), dolor (pain) and functiolaesa (disturbed function).

Cytokines are the physiological messengers of the inflammatory response. The principal molecules involved are tumor necrosis factor α (TNF-α), interleukins, interferons and colony stimulating factors. Polymorphonucleocytes (PMNs), monocytes / macrophages and endothelial cells are the principal cells involved in the inflammatory response. Leukocyte activation leads to increased leukocyte aggregation and tissue infiltration within the microcirculation where these leukocytes (PMNs and macrophages) undergo a respiratory burst, and increase their oxygen consumption and production of cytokines and other inflammatory mediators such as nitric oxide [8].

Localized inflammation is a physiological protective response, which is generally tightly controlled by the body and usually limited to the site of injury. Loss of this local control or an overly activated response results in an exaggerated systemic response, a phenomenon which is clinically identified today as the systemic inflammatory response syndrome (SIRS) [8]. SIRS may be initiated by infection or by non-infectious causes such as trauma, auto-immune reactions, cirrhosis and pancreatitis. SIRS may result in organ dysfunction, which can progress to MODS [8]. The first descriptions of MODS have emphasized its common association with occult or poorly controlled infection [9]. However, our laboratory was the first to suggest that a generalized inflammatory response, rather than infection per se, is the cause of MODS [10]. Although

common in patients with MODS, infection is not necessarily present and frequently follows rather than precedes the development of the syndrome [2,11]. Although it is difficult to differentiate the clinical manifestations of inflammation from the infections that are commonly their cause, it can be shown that the severity of the inflammatory response rather than the presence or absence of infection is the more important determinant of ICU survival [12]. Cohort studies in critically ill patients show that increased circulating levels of pro-inflammatory cytokines such as TNF-α and IL-6 in response to inflammatory stimuli are associated with organ dysfunction and an increased risk of death [13-16]. Although the synthesis and release of biochemical mediators of inflammation is characteristic of both experimental and clinical sepsis, the mechanisms which cause these molecules to induce organ injury are largely unknown. In severe sepsis, proinflammatory cytokines exert many biological effects, such as activation of coagulation and enhanced formation of thrombin and fibrin clots. So far, activated protein C, an endogenous fibrinolytic agent, represents the only specific therapeutic modality apart from antibiotics that has been shown to improve mortality in severe sepsis and septic shock [6].
Until we gain a better understanding in this field, our ability to devise rational and effective therapeutic interventions will be seriously constrained. Intervention studies in humans will probably not be very successful before pre-clinical experimental studies and animal studies have provided a better fundamental insight in the molecular and cellular mechanisms that lead to MODS.

AIM OF THE STUDY

Investigations into the mechanisms of MODS necessitate the collection of tissue samples and invasive procedures that cannot be performed in humans. As a substitute, an animal model for MODS has been developed by our research

group. In 1986, Goris and coworkers have described a model that has been recognized as the only model so far which shares a number of characteristics with human MODS [11]. It is called the zymosan induced generalized inflammation (ZIGI) model and has been adopted by other research groups as well. Chapter 2 provides an overview of the scientific literature concerning the ZIGI model.

The purpose of the thesis was to examine mediators and mechanisms involved in the pathogenesis of MODS in mice. The following questions have been addressed:

-What is the expression of mRNA for a number of inflammatory cytokines in various organs during the development of MODS (chapter 3)?

-Can MODS progress in animals without endogenous TNF-α (chapter 4)?

-Can treatment with pentoxifylline, a compound that inhibits the production of TNF-α and possibly that of other pro-inflammatory cytokines, mitigate the development of MODS (chapter 5)?

-Is nitric oxide, produced by inducible nitric oxide synthetase (iNOS), an essential mediator in the development of organ damage (chapter 6)?

-Are enzymes from the matrix metalloproteinases (MMP) family upregulated in the various organs during evolving organ damage (chapter 7)?

References

1. Seely AJ, Christou NV. Multiple organ dysfunction syndrome: exploring the paradigm of complex nonlinear systems. Crit Care Med 2000; 28:2193-2200.

2. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit Care Med 2001; 29:S99-106.

3. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med 1992; 20:864-874.

4. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. Ann Surg 1992; 216:117-134.

5. Regel G, Grotz M, Weltner T, Sturm JA, Tscherne H. Pattern of organ failure following severe trauma. World J Surg 1996; 20:422-429.

6. Sharma S, Kumar A. Septic shock, multiple organ failure, and acute respiratory distress syndrome. Curr Opin Pulm Med 2003; 9:199-209.

7. Beal AL, Cerra FB. Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction [see comments]. JAMA 1994; 271:226-233.

8. Davies MG, Hagen PO. Systemic inflammatory response syndrome. Br J Surg 1997; 84:920-935.

9. Baue AE. Multiple, progressive, or sequential systems failure. A syndrome of the 1970s. Arch Surg 1975; 110:779-781.

10. Goris RJ, te-Boekhorst TP, Nuytinck JK, Gimbrere JS. Multiple-organ failure. Generalized autodestructive inflammation? Arch Surg 1985; 120:1109-1115.

11. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

12. Marshall J, Sweeney D. Microbial infection and the septic response in critical surgical illness. Sepsis, not infection, determines outcome. Arch Surg 1990; 125:17-22.

13. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, Suter PM. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. Crit Care Med 1996; 24:392-397.

14. Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, Lamy M. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann Surg 1992; 215:356-362.

15. Panacek EA, Kaul M. IL-6 as a marker of excessive TNF-alfa activity in sepsis. Sepsis 1999; 3:65-73.

16. Stuber F, Petersen M, Bokelmann F, Schade U. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. Crit Care Med 1996; 24:381-384.

2

Zymosan-induced generalized inflammation in mice: experimental studies into mechanisms leading to the multiple organ dysfunction syndrome.

Volman,TJH; Hendriks,T; Goris,RJA

Shock. 2005; in press

ABSTRACT

Patients suffering from the multiple organ dysfunction syndrome (MODS) comprise a heterogeneous population, which complicates research in its pathogenesis. Elucidation of the mechanisms involved in the development of MODS will ultimately necessitate the collection of tissue samples and the performance of invasive procedures. These requirements greatly reduce the possibilities for research in human subjects. Therefore, an animal model for MODS is a necessary and valuable tool. In the mid-1980s, the zymosan-induced generalized inflammation (ZIGI) model was introduced. Intraperitoneal injection of zymosan in mice or rats leads, in the course of 1-2 weeks, to increasing organ damage and dysfunction. This model has been recognized as the one which features best resemble human MODS and it has been used widely to study systemic inflammation in relation to organ failure. This review describes the ZIGI model and gives an overview of the results obtained.

INTRODUCTION

The Multiple Organ Dysfunction Syndrome (MODS) became a problem in intensive care units (ICUs) after improvements in hemodynamic support and fluid resuscitation led patients to survive the acute phase of shock and sepsis. Thirty years after its initial description [1], the mortality of MODS remains essentially unchanged. MODS still is the principal cause of morbidity and mortality for patients admitted to an ICU. The costs of its treatment are enormous [2,3].

MODS has been documented to occur after a number of diverse clinical conditions, such as infection, shock, trauma and pancreatitis. Patients suffering from MODS thus comprise a heterogeneous population, which complicates research into its pathogenesis. Still, the development of rational interventions requires insight in the pathological processes involved. Elucidation of the mechanisms involved in the development of MODS will ultimately necessitate the collection of tissue samples and the performance of invasive procedures. These requirements greatly reduce the possibilities for research in human subjects. Therefore, an animal model for MODS is a necessary and valuable tool. In 1986, Goris and coworkers [4] have first described a model that has subsequently been recognized as the one which features best resemble human MODS. This review describes the model and aims to arrange the results reported in a variety of studies.

ZYMOSAN

Zymosan is a substance derived from the cell wall of the yeast *Saccharomyces cerevisiae*. It is composed of polysaccharide chains of various molecular weight, containing approximately 73% polysaccharides, 15% proteins, 7% lipids and inorganic components [5]. When injected into animals, it induces inflammation by

inducing a wide range of inflammatory mediators. These include activated components of the complement system [6], prostaglandins and leukotrienes [7], platelet aggregation factor [8], oxygen radicals [9] and lysosomal enzymes [10]. Also, zymosan directly activates macrophages [11]. Toll-like receptor (TLR)-2 has been shown to be involved in zymosan-induced signaling [12]. After zymosan is phagocytosed, macrophages release lysosomal enzymes [13], reactive oxygen metabolites, arachidonic acid [14] and tumor necrosis factor-α (TNF-α) [15]. Because zymosan is not degradable, phagocytosis by macrophages results in a prolonged inflammatory response. This effect can be enhanced by the use of mineral oil as a carrier.

THE ZYMOSAN-INDUCED GENERALIZED INFLAMMATION (ZIGI) MODEL

Most experimental models of critical illness, such as infusion of endotoxin or bacteria or cecal ligation and puncture, are essentially acute models for sepsis. Almost invariably, they are used to improve or investigate short-term mortality and organ failure. The observation period consists of hours rather than days. Since it usually requires at least 5-7 days after the initial event to fully develop MODS in critically ill patients, chronic models are likely to be more relevant to the human situation. To date, the ZIGI model is the only long-term experimental animal model for MODS.

Intraperitoneal injection of zymosan in a high dosage (0.8-1.0 mg/ g body weight) in rats and mice results in a three-phasic illness. After the injection of zymosan the animals develop an acute peritonitis. They are very ill during the first two days, as reflected by a ruffled fur, diarrhea, lethargic behavior and a decrease in body weight. During this phase, the animals are leukopenic and oxygen consumption [4], myeloperoxidase levels (indicating neutrophil recruitment) in lungs and peritoneum [16,17] and endothelial permeability [18] are

increased. Bacterial translocation to the peritoneal cavity and mesenteric lymph nodes is found regularly [19]. The mortality in this phase is usually between 10 and 35%. Because the early mortality suddenly increased dramatically a few years ago (up to 100%), most likely as the result of changing conditions within our central animal facility, we have introduced a small modification to the initial model [20]. This modification consists of an aseptic intraperitoneal injection of 40 µg lipopolysaccharide (LPS) six days before the intraperitoneal injection of zymosan in mice. LPS activates a variety of cell types, such as monocytes/macrophages, endothelial cells and some epithelial cells [21]. Triggering the immune system by a small dose of LPS prevents an excessive response to zymosan six days later, because the mortality rate of the animals in the first days after zymosan returned to normal (below 30%).

In the second phase of the model (day 3 to 5) the condition of the surviving animals appears to return to normal, as reflected by the display of natural curiosity, grooming, a smooth fur, and disappearance of diarrhea. Body temperature is restored to normal and animals gain weight again. There is no mortality in this phase. From approximately day 7 onwards, animals enter a MODS-like phase, characterized by lethargic behavior, breathing difficulties, progressive weight loss, and a substantial decrease in body temperature. Between day 7 and day 14 the mortality is between 20 and 30%.

Histopathological changes in various organs indicate a gradual development of a generalized inflammatory response with extensive tissue injury [22]. Massive hemorrhages develop in the lungs. The liver displays progressive accumulation of macrophage-like and mononuclear cells, forming granuloma-like structures. The spleen displays great changes in red and white pulp with increased numbers of megakaryocytes and plasma-like cells [23]. Organ damage in this phase is also reflected by an increase in the wet weight of lung, liver, and spleen [23], indicating tissue oedema. Very recently, we have found that the activity of matrix metalloproteinases (MMPs), which class of enzymes catalyzes connective tissue

degradation, is upregulated during the development of organ damage [24]. During the third phase, the course of body weight and body temperature –and, obviously, mortality- are quantitative measures of illness. Figure 1 shows the course of these parameters during a typical experiment. At the end of the experiment, organ weight or (semi-) quantitative histology yield good parameters for organ disease.

BACTERIA

Initially, MODS was believed to occur only after a bacterial infection or endotoxemia. Zymosan causes an intense inflammatory response without administration of exogenous bacteria or modification of the normal bacterial flora. This allows examination of the possible role of bacterial translocation in the development of MODS. Studies in rats have shown that in the ZIGI model bacterial translocation is common [19,22,25-28] but not essential for the development of organ damage. Using germ-free rats [4] and selective decontamination of the gastrointestinal tract of normal rats [25], Goris et al. have demonstrated that zymosan is capable of inducing MODS in the absence of bacteria. In streptomycin-decontaminated rats, survival was 100% despite massive translocation of streptomycin-resistant enterobacteriacea [29]. Streptomycin has been shown to prevent macrophage activation [30]. Also, it has been reported that mice that are hyporesponsive to endotoxin remain only in a slightly better condition than endotoxin-sensitive mice [31], which suggests that endotoxin is not essential in the pathogenesis of MODS. Elimination of macrophages lowers the systemic toxic response (as indicated by the degree of conjunctivitis, ruffled fur, lethargy and diarrhea) and subsequent mortality, despite the fact that systemic bacterial translocation is enhanced [32]. Taken together, these observations support the hypothesis that, in this model, MODS is the result of a generalized inflammatory response, rather than infection per se.

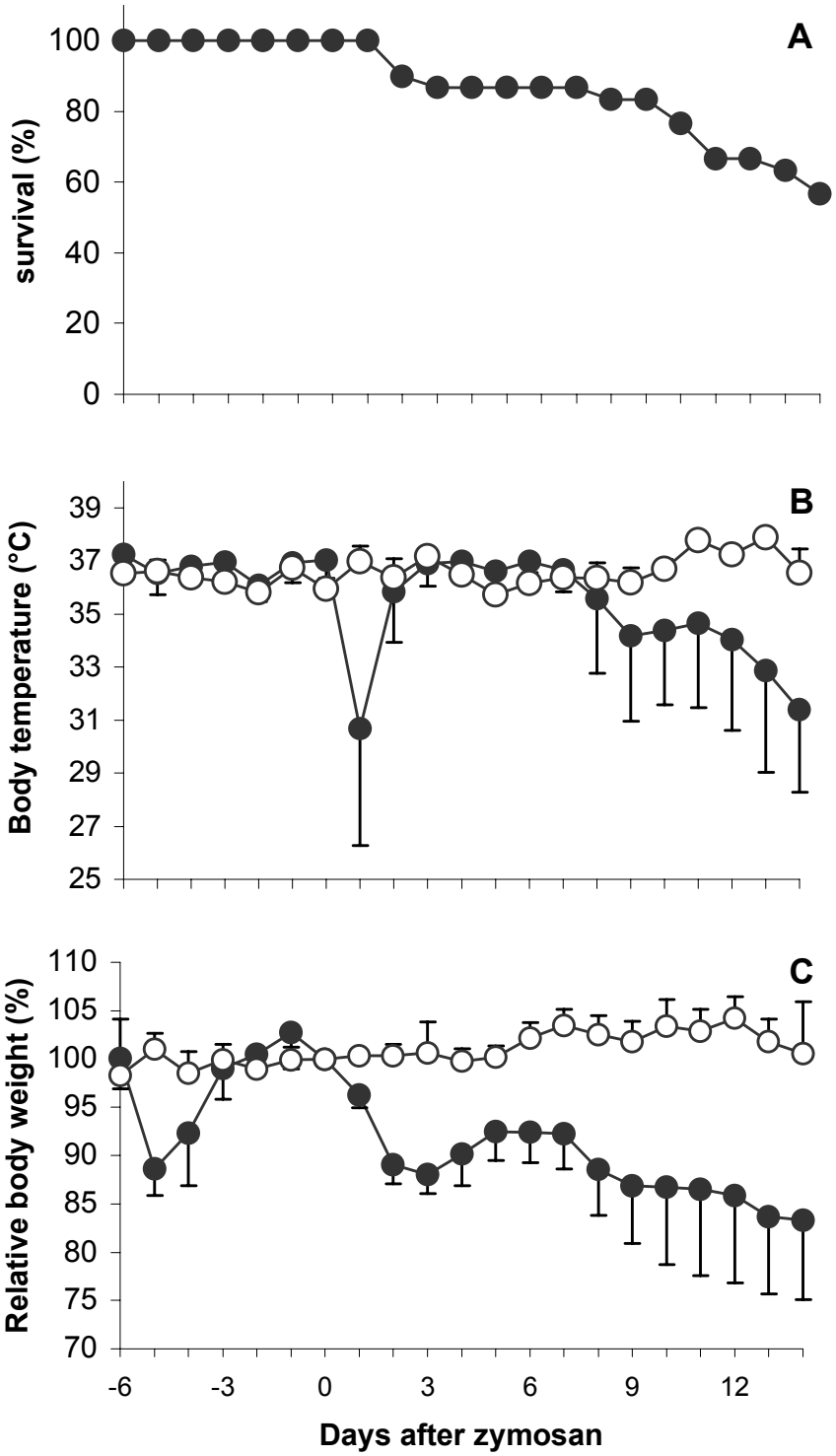

*Figure 1. Survival and clinical symptoms in the ZIGI model. Survival (A), body temperature (B) and relative body weight (C) of C57BL/6 mice (closed circles, n=30) in comparison with untreated control mice (open circles, n=6). Day –6: LPS i.p.; day 0: zymosan i.p. Relative body weight was calculated as (body weight/body weight at day 0)*100%. Data represent average values + SD.*

CYTOKINES

Since MODS is now believed to be the result of a generalized inflammatory response, increasing attention has been paid to the role of pro-inflammatory mediators in its pathophysiology. The (over)expression of these mediators, which include cytokines, complement factors and reactive oxygen species, results in increased peripheral dilatation, leukocyte/endothelial cell activation, excessive microvascular permeability and accelerated microvascular clotting [33]. These responses contribute to the development of profound changes in the various organs, which may eventually lead to MODS [34,35].

Zymosan induces secretion of TNF-α, IL-1β, IL-6 and IL-8 by macrophages [36]. Three mechanisms are known by which zymosan can trigger cytokine secretion. Firstly, normal serum contains IgG immunoglobulins that bind to zymosan [37]. The complexes formed this way activate the classical pathway of the complement system, and may induce TNF-α release by cross-linking macrophage Fc receptors [38]. Secondly, zymosan activates complement via the alternative pathway [39]. The subsequent binding of C3b/iC3b to zymosan particles enables uptake of the particles via complement receptors on macrophages and granulocytes. Thirdly, the binding of C3b to the zymosan surface results in increased conversion of C5 to C5a [40], which triggers TNF-α release [41]. Whereas these pathways act synergistically to induce TNF-α release [42], the mechanisms by which other cytokines are induced by zymosan are less clear. Probably IL-1β and IL-6 release by macrophages is partly dependent on exposure to TNF-α. A strong increase in the secretion of IL-8 is induced by phagocytosis of zymosan by monocytic cells [43].

In zymosan-induced organ damage, TNF-α appears to play an important role. Jansen et al. [44] found that plasma TNF-α and interleukin (IL)-6 concentrations were transiently increased during the first 24 hours after administration of zymosan. After eight days a prominent peak of biologically inactive TNF-α was

observed. There also was a significantly enhanced LPS-stimulated production of TNF-α by peritoneal cells over the entire experimental period. The plasma TNF levels did not correlate with the peritoneal macrophage TNF production levels, especially in the second phase of illness, when no plasma TNF could be detected. Peritoneal macrophage TNF production was very high. Volman et al. [45] found an increased expression of TNF-α, IL-1β, IL-6, IL-12 and interferon- γ (IFN-γ) mRNA in a time-dependent pattern in several organs. This increase was most pronounced for TNF-α in the lungs and liver. Moreover, in TNF-α/lymphotoxin-α knockout mice [20] and in TNF receptor p55 knockout mice [46] both survival and clinical condition were significantly improved after zymosan challenge if compared to the respective wildtype controls. Also, intervention studies directed at TNF-α using either a specific antibody [47], chlorpromazine [48], IL-10 [49,50] and the alkaloid fangchinoline [51], have demonstrated mitigation of zymosan-induced MODS. This was reflected in improved survival, a lesser increase in organ weight, a less severe drop in body temperature and –weight, improved pulmonary compliance and reduced hemorrhage formation in the lungs. Although all these data suggest an important role for TNF-α in the development of organ damage, it is unlikely to be the only mediator responsible since interventions directed against TNF-α alone or even its complete absence could reduce but not prevent the symptoms of zymosan-induced MODS.

THE COMPLEMENT SYSTEM

Excessive complement activation has been proposed to play a role in MODS [52]. Complement factor C5, participating in both the classical and alternative pathway, is a potent polymorphonuclear neutrophil (PMN) chemoattractant. PMNs are capable of producing a variety of inflammatory mediators, including cytokines, oxygen radicals, proteases and arachidonic acid metabolites, thereby possibly contributing to the tissue damage observed in MODS.

Nieuwenhuijzen et al.[53] injected C5 deficient mice and C5 sufficient mice with zymosan and observed a lower mortality and a less severe decrease in body temperature and body weight in the first, acute, phase in the C5 deficient mice. Also, the C5 deficient mice scored better on a symptom scale of lethargy, conjunctivitis, diarrhea and ruffled fur in this phase. However, deficiency of C5 could not prevent organ damage (assessed as the relative organ weights) in the late, MODS-like, phase. Miller et al.[54] also injected C5 deficient mice and C5 sufficient mice with zymosan, and found a lower mortality in both phases in C5-deficient mice. However, in a follow-up study [55], the same research group did not find significant differences in pulmonary neutrophil infiltration between the two types of mice in the first phase, although the absence of C5 caused a decrease in circulating neutrophil levels.

In vivo studies with complement-modulating substances have also been performed. Ivanovska et al.[56] observed that administration of complement-inhibiting bisbenzyisoquinoline alkaloids to zymosan-treated mice yielded a lower mortality and a lower percentage of mice with injury of liver, kidney and spleen. The latter was assessed from the presence of macroscopically observed fibrosis, solid accretions around these organs, foci of inflammation on renal membranes and enlargement of the spleen.

Taken together, these studies suggest a certain involvement of complement factors in the development of murine MODS. However, since the number of studies is limited and the results are not unequivocal, it remains to be established if their role is essential and causative.

OXIDANTS

The release of oxidants by activated phagocytic cells can result in severe cell damage, most notably the peroxidation of cell membrane lipids. The lipid peroxidation process is associated with impaired cell membrane function and

decreased adenosine triphosphate production, eventually leading to cell lysis and increased vascular permeability. Because of their cytotoxic effects, reactive oxygen and nitrogen species have been implicated in the pathogenesis of MODS [33,34]. Numerous studies have been undertaken to study the role of these substances in the ZIGI model.

The occurrence of lung and liver lipid peroxidation was found to correlate well with the triphasic illness. Lipid peroxidation is elevated in both the first [57,58] and the third phase [58] of illness. The production capacity of superoxide (O_2^-) and nitric oxide (NO) by peritoneal macrophages increases from day 7 onwards and thus coincides with the occurrence of organ damage [59]. Also, several studies have shown the presence of nitrotyrosine, a biomarker of the reactive nitrogen species peroxynitrite, in the course of the ZIGI model [60-63]. Demling et al. [64,65] reported an increased oxidant activity in the lung and a reduced antioxidant capacity in the liver.

Intervention studies directed at counteracting oxidant stress have also been performed. Van Bebber et al. [66] administered a combination of superoxide dismutase and catalase to rats treated with zymosan and found that, while lipid peroxidation was significantly decreased, mortality and organ damage remained unchanged. Since the rats were sacrificed 48 h after zymosan, data are limited to the first, acute, phase. Long-term administration of hydroxyl scavengers (mannitol, dimethylsulfoxide, dimethylthiourea) did improve neither the course of body temperature and body weight, nor the respiratory rate, organ weights or mortality [29]. Mainous et al. [27] found that pre-treatment of rats and mice with allopurinol or ibuprofen, which are both inhibitors of oxygen-derived free radical production, provided protection against bacterial translocation 6 h after zymosan injection and improved the survival rate, as measured after 7 days. Tempol, a small molecule that permeates biological membranes and scavenges superoxide anions and other free radicals, attenuated organ damage, as observed histologically in lung, liver and intestine, and subsequent mortality in a study

with rats [63]. Cuzzocrea et al. [67] reported that intraperitoneal injection of N-acetylcysteine, a glutathione precursor, prevented the development of peritonitis and reduced peroxynitrite formation in a dose-dependent manner when given to zymosan-shocked rats. In addition, this intervention was effective in preventing the development of lung and intestinal injury and neutrophil infiltration. *In vitro* studies have demonstrated that oxidative injury in various cell types is related in part to DNA single strand breakage and the subsequent activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS). [68]. Massive ADP ribosilation of nuclear proteins by PARS can result in cellular energy depletion and injury [69] and, ultimately organ damage. Several studies have shown that inhibition of PARS prevents neutrophil recruitment and reduces microscopically visible organ injury elicited by intraperitoneal injection of zymosan. [70-72].

An enzyme that has received special attention regarding tissue damage is inducible nitric oxide (NO) synthase (iNOS). It is induced by cytokines and LPS and generates NO, a reactive nitrogen species that directly and indirectly may induce tissue damage during critical illness [73]. NO is also produced by the constitutive isoforms of nitric oxide synthase, but only in relatively small amounts which are thought to be harmless though of great importance to normal physiology [73]. Cuzzocrea et al. have reported experiments that suggest a causative role for NO in zymosan-induced organ damage. Administration of L-NAME, a nonselective NOS inhibitor, attenuated zymosan-induced acute peritonitis in rats [74,75]. Also, iNOS knockout mice showed a lower mortality and a lesser degree of microscopically visible organ damage and neutrophil infiltration in the lungs, liver and intestine than wildtype mice [61]. Also, administration of aminoguanidine, a selective iNOS inhibitor, to wildtype mice had similar effects. However, these beneficial effects in aminoguanidine-treated mice and iNOS knockout mice could not be confirmed by Volman et al. [76]. This apparent discrepancy might be explained by the fact that the latter authors examined the course of illness for at least 14 days, thus essentially investigating

MODS after a chronic systemic inflammation. In contrast, Cuzzocrea et al. limited their observations to the first 48 h after zymosan administration, thus effectively studying nonseptic shock.
Taken together, it is clear that in the course of the ZIGI model there is increased oxidant activity. Still, intervention studies aimed to suppress oxidant stress have not demonstrated complete protection against the development of MODS. This means that, although oxidants certainly contribute to organ damage, other mechanisms must do so as well.

INTERVENTIONS DIRECTED AT MULTIPLE MEDIATORS

Since it appears unlikely that MODS is the result of the overexpression of a single mediator, interventions directed at multiple mediators may be expected to yield better results than those aimed at a single compound. Although dietary fish oil is believed to possess anti-inflammatory effects, intervention studies with dietary fish oil did not prevent MODS and did not reduce the expression of TNF-α and IL-6 [77,78]. Cuzzocrea et al. [62] reported the effects of hyperbaric oxygen therapy on multiple organ failure induced by zymosan in the rat. Although the precise molecular mechanisms remain unclear, it appeared that hyperbaric oxygen therapy exerts potent anti-inflammatory effects on systemic inflammation and effectively prevents the development of lung, liver and intestinal injury. However, the experiment was ended after 72 hours, when the degree of organ damage was only modest.
Tyrosine kinase inhibitors can reduce the formation and/or effects of TNF-α and IL-1β, the expression of iNOS and cyclooxygenase-2 and the activation of the transcription factor NF-κB [79,80]. Dugo et al. [81] studied the effects of the tyrosine kinase inhibitor tyrphostin AG126. This compound apparently attenuates the severity of MODS and reduces the mortality induced by zymosan. Still, Volman et al. did not find any beneficial effects of pentoxifylline, a substance that, at

least *in vitro*, can reduce the expression of TNF-α, IL-1β and IL-6 (manuscript in preparation).

Inhibition of NF-κB appears to be a promising approach to prevent the development of zymosan induced MODS. Cuzzocrea et al. [82] found that inhibition of calpain I, which is an indirect way to prevent NF-κB activation, results in a lesser degree of inflammatory organ damage and a lower mortality during zymosan induced MODS in rats. In apparent contrast, recent experiments in our laboratory failed to demonstrate any beneficial effects on morbidity and mortality of pyrrolidine dithiocarbamate (PDTC), a non-specific NF-κB inhibitor, in zymosan-treated mice (Volman et al., manuscript in preparation).

CONCLUSIONS AND FUTURE DIRECTIONS

Figure 2 summarizes the mechanisms which are currently thought to contribute to the development of MODS. The ZIGI model has contributed significantly to our understanding of its pathophysiology, especially in the areas which are highlighted in the scheme. However, as any animal model it has its limitations. Animal models are suitable to investigate the mechanisms of disease. They are also necessary for the preclinical testing of potential therapeutic agents. However, it remains difficult to extrapolate the results of such studies to clinical practice, because, for instance, the study population in animal studies is very homogenous (same sex and age, inbred population), whilst patient populations usually are very heterogeneous. This does not in any way diminish the fact that animal studies can yield valuable indications for treatment options and, if an agent is proven successful in multiple animal models, there is a reasonable chance that it will be effective in patients as well. In the past, many efforts have been undertaken to identify the single mediator that causes most or all of the trouble in generalized inflammation. It now appears that MODS is unlikely to be the result of the overexpression of a single mediator. Intervention studies

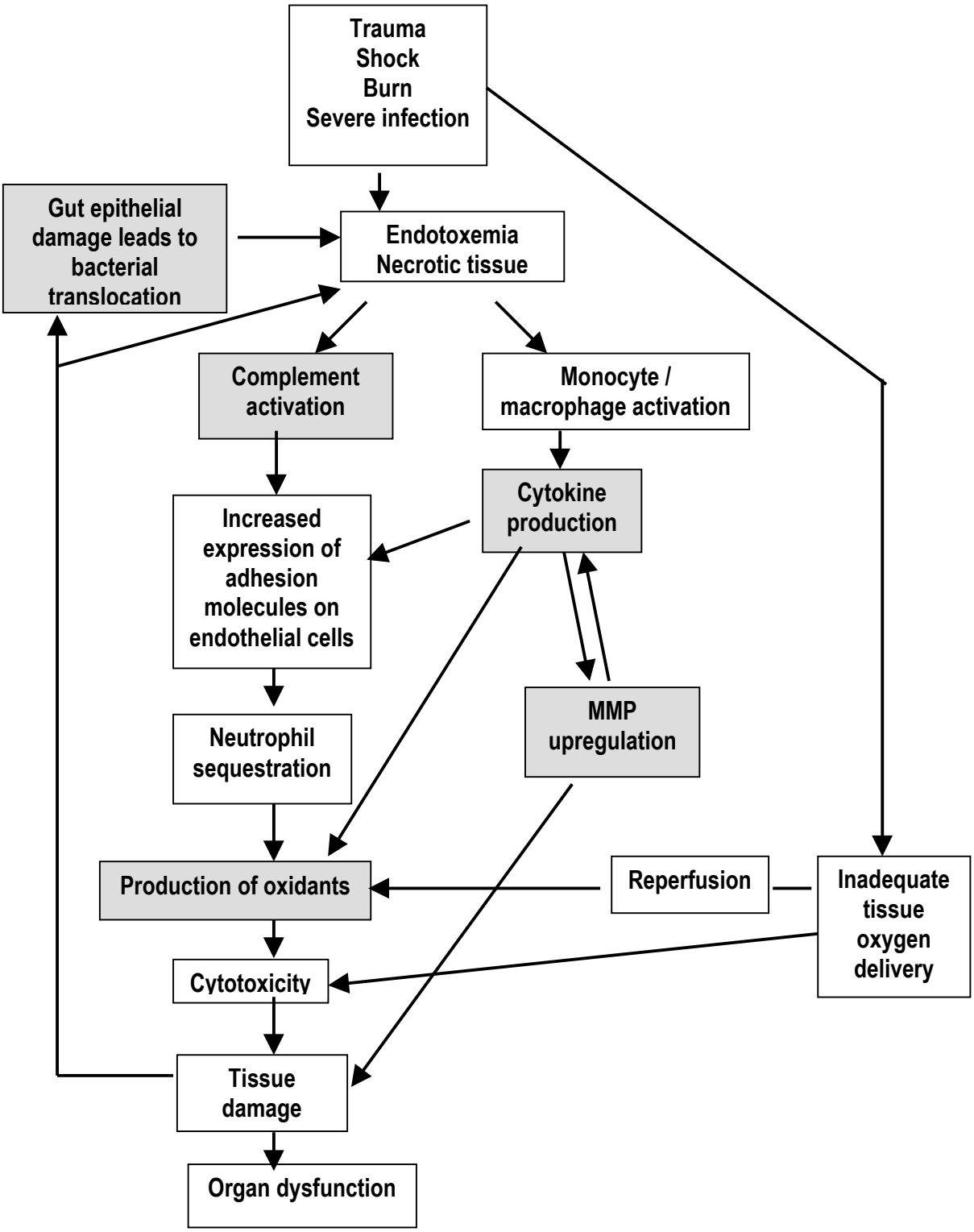

Figure 2. Clinical and cellular events leading to the development of MODS. The shaded areas highlight the mechanisms where experimental studies using the ZIGI model have contributed significantly to our knowledge.

directed at blocking a single mediator, or a single process, have been successful in quite a number of cases but did not result in a complete disappearance of MODS-like symptoms. Therefore, interventions directed at multiple mediators currently seem the most promising approach. A major problem in performing these studies is discovering or designing agents that can block or modulate multiple mediators in a specific way. For instance, inhibiting the expression of multiple proinflammatory cytokines by inhibiting NF-κB seems a logical approach, but so far very few specific NF-κB inhibitors have been available for use *in vivo*. Next to this practical problem, there appears to be a great risk that inhibition of NF-κB will have harmful side-effects, such as apoptosis of tissues and an impaired defence to bacteria [83].

So far, studies directed at multiple mediators have been performed using protocols that were unspecific, and the mechanisms of actions hardly understood, which makes it difficult to draw definite conclusions from these studies. Hopefully, far more specific interventions will become possible in the near future. This will improve the quality of the studies and will further improve our understanding of the underlying pathophysiology of MODS.

References

1. Tilney NL, Bailey GL, Morgan AP. Sequential system failure after rupture of abdominal aortic aneurysms: an unsolved problem in postoperative care. Ann Surg 1973; 178:117-122.

2. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy [see comments]. Ann Surg 1992; 216:117-134.

3. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit Care Med 2001; 29:S99-106.

4. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

5. Fitzpatrick FW, DiCarlo FJ. Zymosan. Annals of the New York Academy of Sciences 1964; 118:233-262.

6. Pillemer L, Ecker EE. Anticomplementary factor in fresh yeast. J Biol Chem 1941; 137:139-142.

7. Humes JL, Sadowski S, Galavage M, Goldenberg M, Subers E, Bonney RJ, Kuehl FAJ. Evidence for two sources of arachidonic acid for oxidative metabolism by mouse peritoneal macrophages. J Biol Chem 1982; 257:1591-1594.

8. Roubin R, Mencia Huerta JM, Landes A, Benveniste J. Biosynthesis of platelet-activating factor (PAF-acether). IV. Impairment of acetyl-transferase activity in thioglycollate-elicited mouse macrophages. J Immunol 1982; 129:809-813.

9. Nauseef WM, Root RK, Newman SL, Malech HL. Inhibition of zymosan activation of human neutrophil oxidative metabolism by a mouse monoclonal antibody. Blood 1983; 62:635-644.

10. Bonney RJ, Wightman PD, Davies P, Sadowski SJ, Kuehl FAJ, Humes JL. Regulation of prostaglandin synthesis and of the selective release of lysosomal hydrolases by mouse peritoneal macrophages. Biochem J 1978; 176:433-442.

11. Underhill DM. Macrophage recognition of zymosan particles. J Endotoxin Res 2003; 9:176-180.

12. Sato M, Sano H, Iwaki D, Kudo K, Konishi M, Takahashi H, Takahashi T, Imaizumi H, Asai Y, Kuroki Y. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. J Immunol 2003; 171:417-425.

13. Kelly BA, Carchman RA. The relationship between lysosomal enzyme release and protein phosphorylation in human monocytes stimulated by phorbol esters and opsonized zymosan. J Biol Chem 1987; 262:17404-17411.

14. Daum T, Rohrbach MS. Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophages via stimulation of a beta-glucan receptor. FEBS Lett 1992; 309:119-122.

15. Sanguedolce MV, Capo C, Bongrand P, Mege JL. Zymosan-stimulated tumor necrosis factor-alpha production by human monocytes. Down-modulation by phorbol ester. J Immunol 1992; 148:2229-2236.

16. Shayevitz JR, Miller C, Johnson KJ, Rodriguez JL. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. Shock 1995; 4:389-396.

17. Rao TS, Currie JL, Shaffer AF, Isakson PC. In vivo characterization of zymosan-induced mouse peritoneal inflammation. J Pharmacol Exp Ther 1994; 269:917-925.

18. Deng X, Wang X, Andersson R. Alterations in endothelial barrier permeability in multiple organs during overactivation of macrophages in rats. Shock 1996; 6:126-133.

19. Mainous MR, Tso P, Berg RD, Deitch EA. Studies of the route, magnitude, and time course of bacterial translocation in a model of systemic inflammation. Arch Surg 1991; 126:33-37.

20. Volman TJH, Hendriks T, Verhofstad AA, Kullberg BJ, Goris RJ. Improved survival of TNF-deficient mice during the zymosan-induced multiple organ dysfunction syndrome. Shock 2002; 17:468-472.

21. Heumann D, Roger T. Initial responses to endotoxins and Gram-negative bacteria. Clin Chim Acta 2002; 323:59-72.

22. Steinberg S, Flynn W, Kelley K, Bitzer L, Sharma P, Gutierrez C, Baxter J, Lalka D, Sands A, van Liew J, et a. Development of a bacteria-independent model of the multiple organ failure syndrome. Arch Surg 1989; 124:1390-1395.

23. Jansen MJ, Hendriks T, Verhofstad AA, Lange W, Geeraedts-LM J, Goris RJ. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

24. Volman TJH, Goris RJA, Lomme RMLM, DeGroot J, Verhofstad AA, Hendriks T. Increased expression of matrix metalloproteinases in the murine zymosan-induced multiple organ dysfunction syndrome. J Pathol 2004; 203:968-975.

25. Goris RJ, van Bebber IP, Mollen RM, Koopman JP. Does selective decontamination of the gastrointestinal tract prevent multiple organ failure? An experimental study. Arch Surg 1991; 126:561-565.

26. Deitch EA, Kemper AC, Specian RD, Berg RD. A study of the relationship among survival, gut-origin sepsis, and bacterial translocation in a model of systemic inflammation. J Trauma 1992; 32:141-147.

27. Mainous MR, Xu D, Deitch EA. Role of xanthine oxidase and prostaglandins in inflammatory-induced bacterial translocation. Circ Shock 1993; 40:99-104.

28. Mainous MR, Ertel W, Chaudry IH, Deitch EA. The gut: a cytokine-generating organ in systemic inflammation? Shock 1995; 4:193-199.

29. Bebber IPTv, Schillings PH, Goris RJ. Decontamination of the gastro-intestinal tract by streptomycin in an experimental model of MOF, reduces mortality by a mechanism independent of the presence of enterobacteriaceae. In Schlag G, Redl H, Siegel JH, Traber DL (eds). Shock, sepsis and organ failure. New York: Springer Verlag; 1990:461-491.

30. Tewari RP, Kugel HL. Suppressive effect of streptomycin on the phagocytic activity of mouse peritoneal macrophages for Histoplasma capsulatum. Mycopathol Mycol Appl 1971; 44:231-240.

31. van Bebber IP, Speekenbrink RG, Schillings PH, Goris RJ. Endotoxin does not play a key role in the pathogenesis of multiple organ failure. An experimental study. Prog Clin Biol Res 1989; 308:419-423.

32. Nieuwenhuijzen GA, Haskel Y, Lu Q, Berg RD, van Rooijen N, Goris RJ, Deitch EA. Macrophage elimination increases bacterial translocation and gut-origin septicemia but attenuates symptoms and mortality rate in a model of systemic inflammation. Ann Surg 1993; 218:791-799.

33. Davies MG, Hagen PO. Systemic inflammatory response syndrome. Br J Surg 1997; 84:920-935.

34. Yao YM, Redl H, Bahrami S, Schlag G. The inflammatory basis of trauma/shock-associated multiple organ failure. Inflamm Res 1998; 47:201-210.

35. Sharma S, Kumar A. Septic shock, multiple organ failure, and acute respiratory distress syndrome. Curr Opin Pulm Med 2003; 9:199-209.

36. Bondeson J, Browne KA, Brennan FM, Foxwell BM, Feldmann M. Selective regulation of cytokine induction by adenoviral gene transfer of IkappaBalpha into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-kappaB independent. J Immunol 1999; 162:2939-2945.

37. Schenkein HA, Ruddy S. The role of immunoglobulins in alternative complement pathway activation by zymosan. I. Human IgG with specificity for Zymosan enhances alternative pathway activation by zymosan. J Immunol 1981; 126:7-10.

38. Debets JM, van der Linden CJ, Dieteren IE, Leeuwenberg JF, Buurman WA. Fc-receptor cross-linking induces rapid secretion of tumor necrosis factor (cachectin) by human peripheral blood monocytes. J Immunol 1988; 141:1197-1201.

39. Czop JK, Austen KF. Properties of glycans that activate the human alternative complement pathway and interact with the human monocyte beta-glucan receptor. J Immunol 1985; 135:3388-3393.

40. Schenkein HA, Ruddy S. The role of immunoglobulins in alternative pathway activation by zymosan. II. The effect of IgG on the kinetics of the alternative pathway. J Immunol 1981; 126:11-15.

41. Okusawa S, Yancey KB, Van der Meer JW, Endres S, Lonnemann G, Hefter K, Frank MM, Burke JF, Dinarello CA, Gelfand JA. C5a stimulates secretion of tumor necrosis factor from human mononuclear cells in vitro. Comparison with secretion of interleukin 1 beta and interleukin 1 alpha. J Exp Med 1988; 168:443-448.

42. von Asmuth EJ, Maessen JG, van der Linden CJ, Buurman WA. Tumour necrosis factor alpha (TNF-alpha) and interleukin 6 in a zymosan-induced shock model. Scand J Immunol 1990; 32:313-319.

43. Friedland JS, Constantin D, Shaw TC, Stylianou E. Regulation of interleukin-8 gene expression after phagocytosis of zymosan by human monocytic cells. J Leukoc Biol 2001; 70:447-454.

44. Jansen MJ, Hendriks T, Vogels MT, Van der Meer JW, Goris RJ. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

45. Volman TJ, Goris RJ, Van der Meer JW, Hendriks T. Tissue- and time-dependent upregulation of cytokine mRNA in a murine model for the multiple organ dysfunction syndrome. Ann Surg 2004; 240:142-150.

46. Burdon D, Tiedje T, Pfeffer K, Vollmer E, Zabel P. The role of tumor necrosis factor in the development of multiple organ failure in a murine model. Crit Care Med 2000; 28:1962-1967.

47. Jansen MJ, Hendriks T, Hermsen R, Van der Meer JW, Goris RJ. A monoclonal antibody against tumour necrosis factor-alpha improves survival in experimental multiple organ dysfunction syndrome. Cytokine 1998; 10:904-910.

48. Jansen MJ, Hendriks T, Knapen MF, van Kempen LC, Van der Meer JW, Goris RJ. Chlorpromazine down-regulates tumor necrosis factor-alpha and attenuates experimental multiple organ dysfunction syndrome in mice. Crit Care Med 1998; 26:1244-1250.

49. Ferrer TJ, Webb JW, Wallace BH, Bridges CD, Palmer HE, Robertson RD, Cone JB. Interleukin-10 reduces morbidity and mortality in murine multiple organ dysfunction syndrome (MODS). J Surg Res 1998; 77:157-164.

50. Jansen MJ, Hendriks T, de Man BM, Van der Meer JW, Goris RJ. Interleukin 10 mitigates the development of the zymosan-induced multiple organ dysfunction syndrome in mice. Cytokine 1999; 11:713-721.

51. Hristova M, Yordanov M, Ivanovska N. Effect of fangchinoline in murine models of multiple organ dysfunction syndrome and septic shock. Inflamm Res 2003; 52:1-7.

52. Roumen RM, Redl H, Schlag G, Zilow G, Sandtner W, Koller W, Hendriks T, Goris RJ. Inflammatory mediators in relation to the development of multiple organ failure in patients after severe blunt trauma. Crit Care Med 1995; 23:474-480.

53. Nieuwenhuijzen GA, Meyer MP, Hendriks T, Goris RJ. Deficiency of complement factor C5 reduces early mortality but does not prevent organ damage in an animal model of multiple organ dysfunction syndrome. Crit Care Med 1995; 23:1686-1693.

54. Miller CG, Cook DN, Kotwal GJ. Two chemotactic factors, C5a and MIP-1alpha, dramatically alter the mortality from zymosan-induced multiple organ dysfunction syndrome (MODS): C5a contributes to MODS while MIP-1alpha has a protective role. Mol Immunol 1996; 33:1135-1137.

55. Mahesh J, Daly J, Cheadle WG, Kotwal GJ. Elucidation of the early events contributing to zymosan-induced multiple organ dysfunction syndrome using MIP-1alpha, C3 knockout, and C5-deficient mice. Shock 1999; 12:340-349.

56. Ivanovska N, Hristova M, Philipov S. Complement modulatory activity of bisbenzylisoquinoline alkaloids isolated from Isopyrum thalictroides--II. Influence on C3-9 reactions in vitro and antiinflammatory effect in vivo. Int J Immunopharmacol 1999; 21:337-347.

57. Demling R, Nayak U, Ikegami K, Lalonde C. Comparison between lung and liver lipid peroxidation and mortality after zymosan peritonitis in the rat. Shock 1994; 2:222-227.

58. van Bebber IP, Boekholz WK, Goris RJ, Schillings PH, Dinges HP, Bahrami S, Redl H, Schlag G. Neutrophil function and lipid peroxidation in a rat model of multiple organ failure. J Surg Res 1989; 47:471-475.

59. Jansen MJ, Hendriks T, Huyben CM, Tax WJ, Van der Meer JW, Goris RJ. Increasing cytotoxic activity and production of reactive oxygen and nitrogen intermediates by peritoneal macrophages during the development of multiple organ dysfunction syndrome in mice. Scand J Immunol 1996; 44:361-368.

60. Girault I, Karu AE, Schaper M, Barcellos-Hoff MH, Hagen T, Vogel DS, Ames BN, Christen S, Shigenaga MK. Immunodetection of 3-nitrotyrosine in the liver of zymosan-treated rats with a new monoclonal antibody: comparison to analysis by HPLC. Free Radic Biol Med 2001; 31:1375-1387.

61. Cuzzocrea S, Mazzon E, Dugo L, Barbera A, Centorrino T, Ciccolo A, Fonti MT, Caputi AP. Inducible nitric oxide synthase knockout mice exhibit resistance to the multiple organ failure induced by zymosan. Shock 2001; 16:51-58.

62. Cuzzocrea S, Imperatore F, Costantino G, Luongo C, Mazzon E, Scafuro MA, Mangoni G, Caputi AP, Rossi F, Filippelli A. Role of hyperbaric oxygen exposure in reduction of lipid peroxidation and in multiple organ failure induced by zymosan administration in the rat. Shock 2000; 13:197-203.

63. Cuzzocrea S, McDonald MC, Mazzon E, Filipe HM, Centorrino T, Lepore V, Terranova ML, Ciccolo A, Caputi AP, Thiemermann C. Beneficial effects of tempol, a membrane-permeable radical scavenger, on the multiple organ failure induced by zymosan in the rat. Crit Care Med 2001; 29:102-111.

64. Demling R, Lalonde C, Youn YK, Daryani R, Campbell C, Knox J. Lung oxidant changes after zymosan peritonitis: relationship between physiologic and biochemical changes. Am Rev Respir Dis 1992; 146:1272-1278.

65. Demling R, Daryani R, Campbell C, Knox J, Youn YK, Lalonde C. The effect of acute nonbacterial dependent peritonitis on lung and liver oxidant stress and antioxidant activity. Surgery 1993; 114:571-578.

66. van Bebber IP, Lieners CF, Koldewijn EL, Redl H, Goris RJ. Superoxide dismutase and catalase in an experimental model of multiple organ failure. J Surg Res 1992; 52:265-270.

67. Cuzzocrea S, Costantino G, Mazzon E, Caputi AP. Protective effect of N-acetylcysteine on multiple organ failure induced by zymosan in the rat. Crit Care Med 1999; 27:1524-1532.

68. Cochrane CG. Mechanisms of oxidant injury of cells. Mol Aspects Med 1991; 12:137-147.

69. Szabo C, Zingarelli B, O'Connor M, Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. Proc Natl Acad Sci U S A 1996; 93:1753-1758.

70. Szabo C, Lim LH, Cuzzocrea S, Getting SJ, Zingarelli B, Flower RJ, Salzman AL, Perretti M. Inhibition of poly (ADP-ribose) synthetase attenuates neutrophil recruitment and exerts antiinflammatory effects. J Exp Med 1997; 186:1041-1049.

71. Cuzzocrea S, Zingarelli B, Costantino G, Sottile A, Teti D, Caputi AP. Protective effect of poly(ADP-ribose) synthetase inhibition on multiple organ failure after zymosan-induced peritonitis in the rat . Crit Care Med 1999; 27:1517-1523.

72. Mazzon E, Serraino I, Li JH, Dugo L, Caputi AP, Zhang J, Cuzzocrea S. GPI 6150, a poly (ADP-ribose) polymerase inhibitor, exhibits an anti-inflammatory effect in rat models of inflammation. Eur J Pharmacol 2001; 415:85-94.

73. Kubes P. Inducible nitric oxide synthase: a little bit of good in all of us. Gut 2000; 47:6-9.

74. Cuzzocrea S, Filippelli A, Zingarelli B, Falciani M, Caputi AP, Rossi F. Role of nitric oxide in a nonseptic shock model induced by zymosan in the rat. Shock 1997; 7:351-357.

75. Cuzzocrea S, Zingarelli B, Sautebin L, Rizzo A, Crisafulli C, Campo GM, Costantino G, Calapai G, Nava F, Di Rosa M, Caputi AP. Multiple organ failure following zymosan-induced peritonitis is mediated by nitric oxide. Shock 1997; 8:268-275.

76. Volman TJH, Goris RJ, van der Jagt M, van de Loo FAJ, Hendriks T. Organ damage in zymosan-induced multiple organ dysfunction syndrome in mice is not mediated by inducible nitric oxide synthase. Crit Care Med 2002; 30:1553-1559.

77. Rosman C, Grond J, Buurman WA, de Smet BJ, de Jonge R, Sietsma M, van Schilfgaarde R, Bleichrodt RP. Fish oil increases the release of tumour necrosis factor and interleukin-6, and has no effect on the incidence of multiple organ failure in rats with peritonitis. Eur J Surg 1993; 159:563-570.

78. Gielen CJ, van As AB, Goris RJ. Do diets enriched with oil prevent multiple organ failure in mice? Eur J Surg 1993; 159:609-612.

79. Novogrodsky A, Vanichkin A, Patya M, Gazit A, Osherov N, Levitzki A. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. Science 1994; 264:1319-1322.

80. Vanichkin A, Patya M, Gazit A, Levitzki A, Novogrodsky A. Late administration of a lipophilic tyrosine kinase inhibitor prevents lipopolysaccharide and Escherichia coli-induced lethal toxicity. J Infect Dis 1996; 173:927-933.

81. Dugo L, Chatterjee PK, Mazzon E, McDonald MC, Paola RD, Fulia F, Caputi AP, Thiemermann C, Cuzzocrea S. The tyrosine kinase inhibitor tyrphostin AG 126 reduces the multiple organ failure induced by zymosan in the rat. Intensive Care Med 2002; 28:775-788.

82. Cuzzocrea S, Chatterjee PK, Mazzon E, Serraino I, Dugo L, Centorrino T, Barbera A, Ciccolo A, Fulia F, McDonald MC, Caputi AP, Thiemermann C. Effects of calpain inhibitor I on multiple organ failure induced by zymosan in the rat. Crit Care Med 2002; 30:2284-2294.

83. Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M. The two faces of IKK and NF-kappa B inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. Nature Med 2003; 9:575-581.

3

Tissue- and time-dependent upregulation of cytokine mRNA in a murine model for the multiple organ dysfunction syndrome.

Volman,TJH; Goris,RJA; Van der Meer,JWM; Hendriks,T

Ann-Surg. 2004 Jul; 240(1): 142-50

ABSTRACT

Objective: To quantitate the course of specific cytokine mRNA expression in tissues that exhibit increasing histopathological changes in time in an animal model for the multiple organ dysfunction syndrome (MODS).
Summary Background Data: The development of treatment protocols for MODS requires elucidation of the mechanisms and mediators involved. In order to devise logical interventions it is necessary to collect data on cytokine expression at tissue level during the development of MODS.
Methods: Ninety-four C57BL/6 mice were given an intraperitoneal injection of 40 μg lipopolysaccharide (LPS), followed by zymosan at a dose of 0.8 mg/g body weight six days later (day 0). Six additional animals did not receive zymosan and acted as controls. At several time points after zymosan injection 6 randomly assigned, zymosan-treated animals were killed and livers, lungs, spleens and kidneys were collected. mRNA expression of TNF-α, IL-1β, IL-6, MIF, IL-12, IFN-γ and IL-10 was measured using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay.
Results: The injection of zymosan induced an acute peritonitis, followed by an apparent recovery. From approximately day 6 onwards, animals started to display MODS-like symptoms. During the peritonitis phase, upregulation of cytokine mRNA was limited. During the period of apparent recovery, cytokine mRNA expression strongly increased, mostly reaching its maximum at day 9 when deterioration of the clinical condition had already set in. The upregulation of TNF-α mRNA was most pronounced, especially in the lungs and liver.
Conclusions: Interventions should preferentially be targeted against multiple cytokines and, at least in this model, there may be a treatment window well after the initial challenge.

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is widely considered to be the leading cause of morbidity and mortality for patients admitted to an ICU. MODS is not a specific disease with a well-characterized pathophysiology but rather a common destructive pathway which may be encountered after a variety of serious insults like major trauma, severe bacterial infection or pancreatitis. It is characterized by a complex and overwhelming host response that usually occurs after massive injury or infection [1]. This overwhelming host response results in systemic inflammation, which is generally believed to be the cause of MODS. Treatment for patients with MODS is still largely supportive. The development of adequate treatment protocols requires elucidation of the mechanisms and mediators involved in its pathophysiology. Lately, many efforts have focused on the identification of endogenous mediators that participate in the inflammatory cascade of MODS [2,3]. Circulating inflammatory mediators are relatively easy to measure, but may be a poor reflection of processes at organ level, where the damage occurs. Since measurements at tissue sites require invasive procedures that cannot be easily performed in humans, our group has developed an animal model [4-6] which is acknowledged as the only one to produce, on a consistent basis, pathological changes that mimic both the sequence and intensity of those occurring in human MODS [7-9]. After a single insult in the form of an intraperitoneal challenge with zymosan rodents suffer an acute, peritonitis-like phase that is followed by an apparent recovery. Thereafter, a gradual deterioration occurs, characterized by increasing organ damage and dysfunction [5]. Previous measurements, in the circulation and on isolated peritoneal cells, have suggested an important role in this process for pro-inflammatory cytokines, especially TNF-α [10]. Although mortality is significantly reduced, TNF-α knockout mice still exhibit MODS-like symptoms indicating the involvement of other mediators [6].

In order to devise logical interventions, possibly directed at multiple cytokines, it is necessary to collect data on their expression at tissue level during the development of MODS. For this purpose, we have performed a comprehensive study quantitating the course of specific cytokine mRNA expression in lung, liver, spleen and kidney, tissues that exhibit increasing histopathological changes in time [5].

A large number of pro-inflammatory mediators have been implicated in the systemic inflammatory response. Specific neutralization of interleukin-(IL)1, TNF-α, interferon-γ (IFN-γ), macrophage migration inhibiting factor (MIF), and IL-12 has been shown to improve survival during murine endotoxemia [11]. Also, studies in critically ill patients have demonstrated that increased circulatory levels of TNF and IL-6 are associated with organ dysfunction [12-15]. During inflammation, IL-10 is also produced. It is regarded as an anti-inflammatory cytokine and its function is probably to mitigate the inflammatory response [16]. To gain insight into the possible role of these cytokines in its pathogenesis, we have measured the expression of TNF-α, IL-1β, IL-6, MIF, IL-12, IFN-γ and IL-10 mRNA in the livers, lungs, spleens and kidneys during the development of MODS in mice.

MATERIAL AND METHODS

Animals

The experiment was performed using 100 C57BL/6 mice, 7 to 9 weeks old, weighing 20-25 g. The animals were fed standard chow (Hope Farms RMB-H, Woerden, The Netherlands) and acidified water ad libitum. The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 5 days. The experiment was approved by the Animal Ethics Review Committee of the University Medical Center Nijmegen.

Zymosan-induced generalized inflammation

The zymosan-induced generalized inflammation (ZIGI) model for MODS has been described previously [4-6]. Briefly, mice were given an aseptic intraperitoneal injection of 40 µg lipopolysaccharide (LPS) (*Escherichia coli*, Sigma Chemical, St Louis, MO) dissolved in 200 µl of phosphate buffered saline (PBS), followed six days later by zymosan administration (day 0). Zymosan A (Sigma Chemical) was sterilized by gamma radiation (5 kGray) and homogeneously suspended in sterile paraffin oil (25 mg/ml) by high frequency vibration during 1 h. After sonification, the suspension was sterilized again in a waterbath at 100°C for 80 min. All suspensions were freshly made before use. Zymosan was given intraperitoneally in a dose of 0.8 mg/g body weight.

Experimental design

From a pool of 100 animals, mice were randomly divided into three groups. Group 1 consisted of 84 mice that were treated with LPS and zymosan. Six additional animals did not receive any experimental treatment and acted as controls (group 2). To monitor the clinical course due to zymosan treatment, a group of 10 additional mice (group 3) was treated with LPS and zymosan and their body temperature, body weight and survival were monitored until day 17, when surviving animals were killed.

At days 2, 5, 7, 9, 11, 13, and 15 after zymosan injection 6 randomly assigned animals from group 1 were killed and livers, lungs, spleens and kidneys were collected. This was also done with the surviving animals at day 17 (11 zymosan-treated mice and the 6 control mice from group 2), when the experiment was ended. Lung damage was assessed macroscopically, using an arbitrary lung score: 0: no hemorrhages 1: hemorrhagic spots covering approximately 1-25%

of the lung surface; 2: confluent hemorrhagic spots covering approximately 25-50% of the lung surface; 3: great areas of hemorrhaging covering 50-75% of the lung surface; 4: completely hemorrhagic lungs (75-100% of the surface covered with hemorrhages).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

After collection, organs were snap-frozen in liquid nitrogen and stored at –80°C. Frozen organs were homogenized and total RNA was extracted with the guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacci [17]. RNA concentration was determined by spectrophotometric measurements with a GeneQuant II RNA/DNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of precipitated total RNA was ascertained by 1% agarose gel electrophoresis.
Isolated RNA was used as a template for the production of first strand cDNA using random hexamers. Relative quantification of target mRNA was performed with a TaqMan real-time RT-polymerase chain reaction (PCR) assay on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Briefly, this method uses the 5'-3' exonuclease activity of *Taq* polymerase to cleave a dually labeled non-extendable TaqMan probe designed to hybridize to a sequence between the forward and the reverse primer. The probe has a quencher dye (6-carboxytetramethylrhodamine) on its 3' end and a reporter dye (6-carboxyfluorescein) at its 5' end. Fluorescence emission of the reporter dye is quenched by the quencher dye until nuclease degradation by *Taq* polymerase during the extension phase of the PCR separates the two dyes and enables detection of the reporter dye fluorescence. The threshold cycle (Ct) is defined as the fractional cycle number at which the reporter fluorescence reaches 10x the standard deviation of the baseline. Quantification of the amount of target in unknown samples is accomplished with measurement of Ct and use of a

standard curve to determine the starting concentration of target. Standard curves for TNF-α, IL-1β, IL-6, MIF and IL-10 were generated using RNA from a mouse macrophage cell line (J774-A1) that was stimulated with LPS (1 μg/ml) for 6 h (TNF-α, IL-1β, MIF) or 4h (IL-6, IL-10), respectively. Standard curves for IL-12 were generated using RNA for J774-A1 cells that were stimulated with LPS (1 μg/ml) and hIFN-γ (10 ng/ml) for 9h. Standard curves for IFN-γ were generated using RNA from murine spleen cells that were stimulated with anti-CD3 (10 μg/ml) for 12h.

In order to correct for variations in cDNA concentration between samples, target gene expression was normalized by dividing the starting concentration of target by the starting concentration of constitutively expressed 18S ribosomal RNA (endogenous control). There were differences in the expression of different target genes in organs of untreated control mice (Table 1). Because in this study we were interested in changes of gene expression during the course of the animal model, the results in figures are represented as upregulations of gene expression. Upregulation of gene expression at any time point was calculated as the median of normalized gene expressions divided by the median of normalized gene expressions of untreated control mice.

Oligonucleotide primers and TaqMan probes were designed with use of the Primer Express 1.0 software (Applied Biosystems Inc). In order to avoid amplification of genomic DNA, cDNA specific primer sets were used when possible (TNF-α, IL-1β, IL-6, IL-12, IFN-γ and IL-10) or, when impossible (MIF), contaminants of genomic DNA was removed from the RNA samples using DNase I (Boehringer Mannheim) before cDNA synthesis. Primers for IL-12 were selected using the gene sequence of the p40 subunit of IL-12. The sequences of primers and probes are represented in table 2.

The PCR conditions were as follows: for amplification of the target product, 25 ng of cDNA (except MIF: 2.5 ng) was added to the PCR mixture consisting of 25 µl TaqMan Mastermix (Applied Biosystems), 12.5 pmol fluorescent probe

Table 1. Constitutive gene expression of target genes in organs of untreated control mice.

	TNF-α	IL-1β	IL-6	MIF*	IL-12	IFN-γ	IL-10
Liver	30.9(2.0)	32.1(1.1)	37.9(1.2)	26.8(1.2)	38.9(1.3)	35.9(2.1)	36.7(1.1)
Lungs	30.7(2.4)	29.6(2.7)	35.3(2.3)	33.3(2.1)	32.9(1.0)	36.3(2.4)	36.9(1.4)
Spleen	28.5(0.2)	26.5(0.7)	35.3(1.2)	28.8(0.7)	29.4(0.9)	31.8(0.3)	32.1(0.7)
Kidneys	36.2(2.6)	32.5(3.1)	40.0(0.1)	23.7(0.7)	35.5(1.9)	37.5(2.1)	39.8(0.3)

Data represent average Ct values and standard deviations between brackets (n=6).
* for MIF, 10 times less cDNA (2.5 ng) was used for the PCR reaction than for other cytokines (25 ng)

Table 2. Sequences (5'-3') of primers and probes used in PCR reactions

target	forward primer	reverse primer	probe
TNF-α	CCCAGACCCTCACACTCAGATC	CCTCCACTTGGTTTGCT	CGAGTGACAAGCCTGTAGCCCACGT
IL-1β	GGACAGAATATCAACCAACAAGTGAT	ATTACACAGGACAGGTATAGATTCTTTCCT	AACGACAAAATACCTGTGGCCTTGGGC
IL-6	TGTATGAACAACGATGATGCACTT	GGTACTCCAGAAGACCAGAGGAAAT	TTTGGTAGCATCCATCAT*
MIF	GCAAGCCCGCACAGTACA	CGTTCGTGCCGCTAAAAGTC	CTGGTCCGGGACCACGTGCACT
IL-12	TCAACATCAAGAGCAGTAGCAGTTC	AGTCCCTTTGGTCCAGTGTGA	CCATTCCACATGTCACTGCCCGAGA
IFN-γ	TGGAGGAACTGGCAAAAGGAT	GCCTGATTGTCTTTCAAGACTTCAA	TGACATGAAAATCCTGCAGAGCCAGATTATC
IL-10	AGTGGAGCAGGTGAAGAGTGATT	TCATGTATGCTTCTATGCAGTTGATG	CAAATTCATTCATGGCCTTGTAGACACCTTG

* Taqman minor groove binder (MGB)-probe

(Applied Biosystems) and 15 pmol of each primer in a final volume of 50 µl. For amplification of the 18S rRNA product, the PCR mixture consisted of 25 µl TaqMan Mastermix and 2.5 µl of pre-developed TaqMan 18S rRNA control kit (Applied Biosystems). Again, the final volume was 50 µl. Thermal cycling conditions were 2 min at 50°C and 10 min of initial denaturation at 95°C to activate *Taq* Gold polymerase, followed by 40 cycles of 2-step PCR consisting of 15 s at 95°C and 1 min at 60°C.

RESULTS

Clinical Course

Survival, body temperature and body weight of mice in group 3 are represented in figure 1. The clinical course of the animal model was similar as described previously [5,6]. Briefly, after the injection of LPS the animals became ill for 1 day, but by the time they received zymosan (day 0) the mice had recovered completely. The injection of zymosan induced an acute peritonitis rendering all animals very ill during the first 2 days, as reflected by a ruffled fur, diarrhea, lethargic behavior, and a decrease in body weight and temperature. During the second phase of the illness (days 3-5), the condition of the surviving animals appeared to return to normal, with an increase in body weight and temperature. From approximately day 6 onward, mice entered the third -MODS-like- phase, indicated by lethargic behavior, breathing difficulties, weight loss, and a decrease in body temperature. The survival curve of mice in group 1 was similar to that of mice in group 3 (Figure 2A). Lung scores of mice in group 1 that were killed at different time points indicate progressive organ damage from day 5 onwards (Figure 2B).

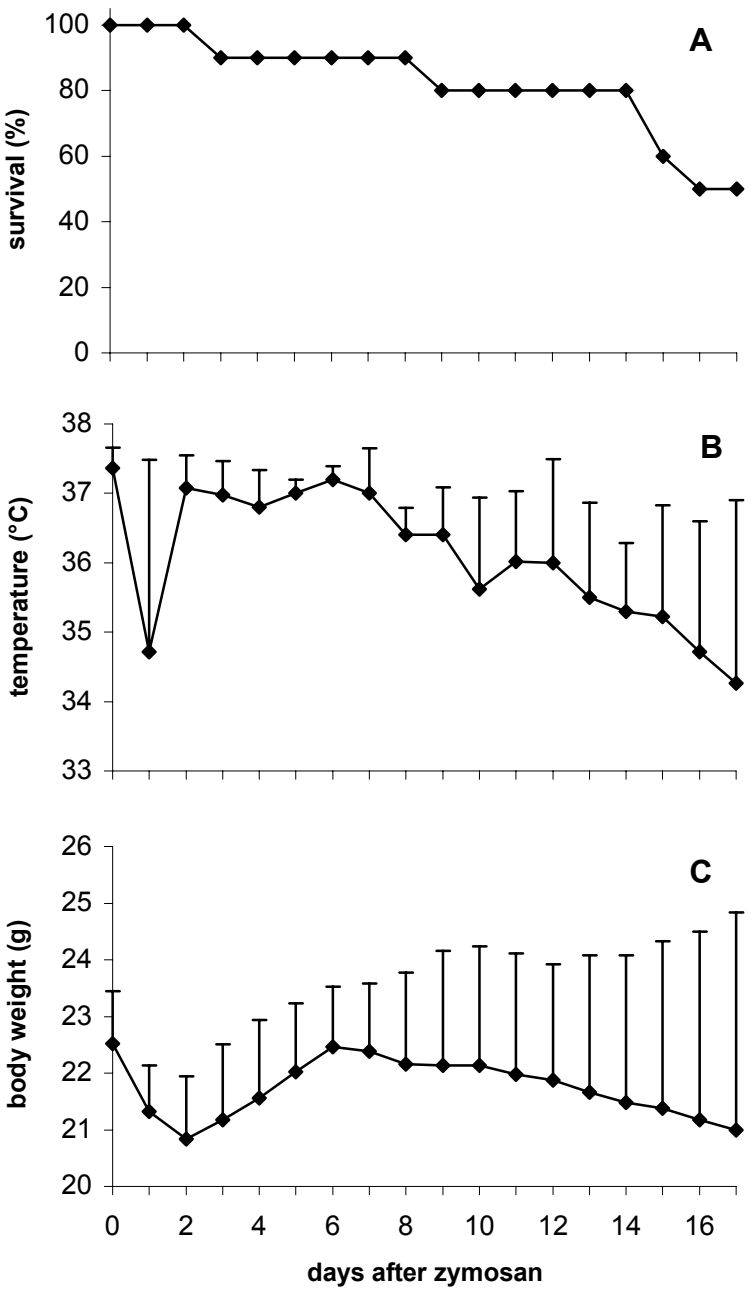

Figure 1. Clinical course after zymosan administration. A group of 10 animals (group 3) was followed to monitor survival (A), body temperature (B) and body weight (C). The latter are depicted as average + SD.

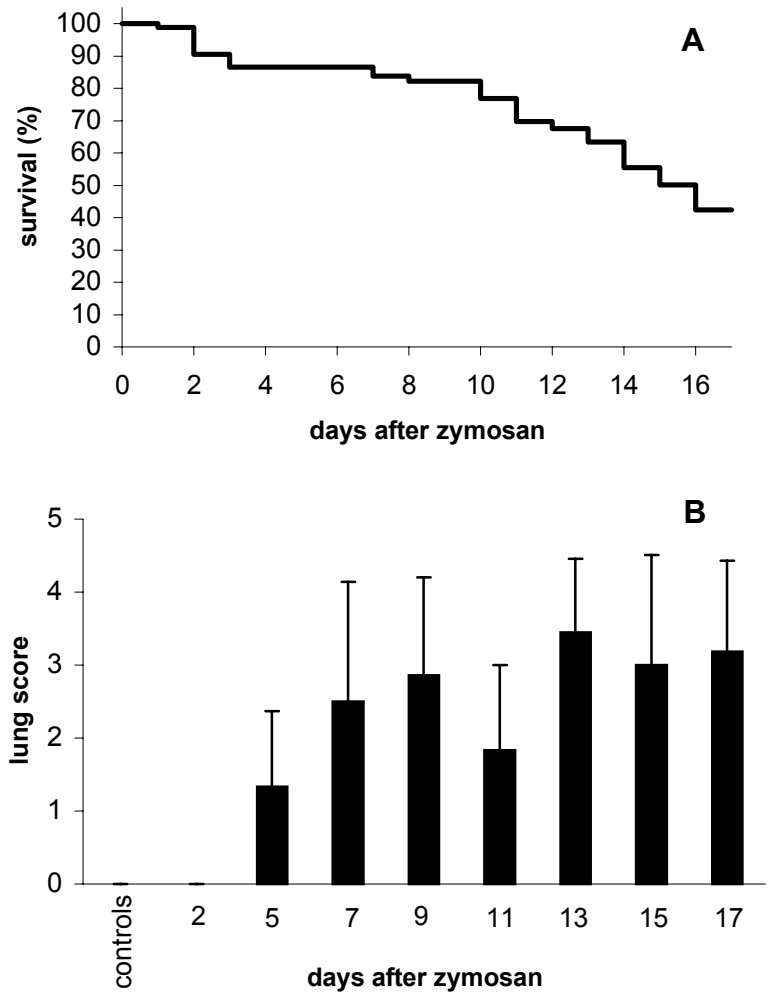

Figure 2. Survival and development of lung damage after zymosan administration. A: Kaplan-Meier survival curve in group 1 (n=84 at day 0), which was used for cytokine mRNA quantitation; B): Lung score for mice in groups 1 and 2 (controls). Bars represent average values (+ SD, n=6 for controls and days 2-15 and n=11 for day 17).

TNF-α mRNA expression

TNF-α mRNA was strongly upregulated in all organs, except for the spleen (Figure 3A). Figure 3 gives, for reasons of clarity, median values only. In order to demonstrate the degree of variation observed between animals, the expression of TNF-α (and IL-1β) mRNA in the liver of all individual animals is illustrated in Figure 4. In the liver, TNF-α message started to increase strongly between days 5 and 7, and peaked around day 9 with a mRNA expression that was 85 times higher than in control animals. After day 9, the expression of TNF-α message in the liver gradually decreased, but remained high until the end of the experiment. In the lungs, the increase in TNF-α message was even higher than in the liver and significant upregulation was observed earlier: at day 2, the increase in TNF-α message was already 16-fold. At day 9, there was a transient and extremely high peak of TNF-α message (237 times the level of controls), but the expression was reduced quickly again over the next 2 days , whereafter the upregulation slowly decreased further. However, it was still substantial at day 17. The upregulation of TNF-α message at day 2 was higher in the kidneys than in the other organs. It peaked again at day 11 and gradually declined afterwards.

IL-1β mRNA expression

The expression of IL-1β message was also substantially increased in all organs, again with exception of the spleen (Figure 3B). In the liver, a strong increase started from day 5 onwards, reaching a peak at day 9, when the expression of IL-1β mRNA was 25 times the level of controls. Upregulation decreased significantly only after day 15 (Figures 3B and 4). In contrast, there was only a limited increase in IL-1β message in the lungs, showing a maximum at day 5. In

the kidneys, however, there was already a remarkable upregulation of IL-1β mRNA at day 2, which reached a peak at day 11 (24-fold).

IL-6 mRNA expression

Significant upregulation of IL-6 mRNA appeared to be limited to liver and kidneys. The expression of IL-6 mRNA in the liver started to increase after day 2 and reached a maximum at days 9 to 11, when the expression was around 15 times the level of controls (Figure 3C); thereafter, the level of mRNA quickly decreased to values approximately 3 times the level of controls. The expression of IL-6 message in the kidneys was upregulated 12-fold at day 2, after which it dropped rapidly.

MIF mRNA expression

In general, the expression of MIF mRNA in the collected organs was very high. However, MIF mRNA was also abundantly expressed in the organs of control animals, as reflected by low Ct values of organs of control mice (Table 1). Therefore, there was no substantial upregulation of MIF mRNA in any organ at any time point (Figure 5A).

IL-12 mRNA expression

The upregulation of IL-12 message in the liver was high: after day 5, the level of mRNA quickly increased to 44 times the level in control animals at day 9 (Figure 5B). Thereafter it slowly subsided but remained significantly elevated until day 17. In neither lungs nor spleen, an increase of IL-12 message was observed. In fact, in the spleen there appeared to be a substantial downregulation

of IL-12 message. In the kidneys, IL-12 mRNA did not change until day 11, when there was a 9-fold increase which slowly disappeared with time.

IFN-γ mRNA expression

There was an increased expression of IFN-γ mRNA in all organs except for the spleen (Figure 5C). In the liver, the mRNA level sharply increased after day 5, and remained high until day 15. In the lungs, the presence of IFN-γ mRNA gradually increased until day 11, where levels were 24-fold upregulated. In the kidneys, the IFN-γ mRNA expression increased from a 6-fold downregulation at day 2 to a 12-fold upregulation at day 7. After that, the level of mRNA fluctuated around 3 times the levels of controls.

IL-10 mRNA expression

In most organs, IL-10 mRNA expression was substantially elevated (Figure 5D). Upregulation was most explicit in the kidneys. However, here the constitutive expression in control animals was extremely low. In the liver, the level of upregulation increased from 4-fold at day 2 to 40-fold at day 9. After that, the IL-10 mRNA expression decreased again. In the lungs, the expression of IL-10 mRNA was also upregulated, but it strongly fluctuated with time. It was maximal at day 15, when the expression was 35 times the level of controls. The IL-10 mRNA expression in the spleen was not much higher than in control animals.

DISCUSSION

The development of MODS in this animal model is accompanied by massive upregulation of the mRNAs for multiple pro-inflammatory cytokines in several

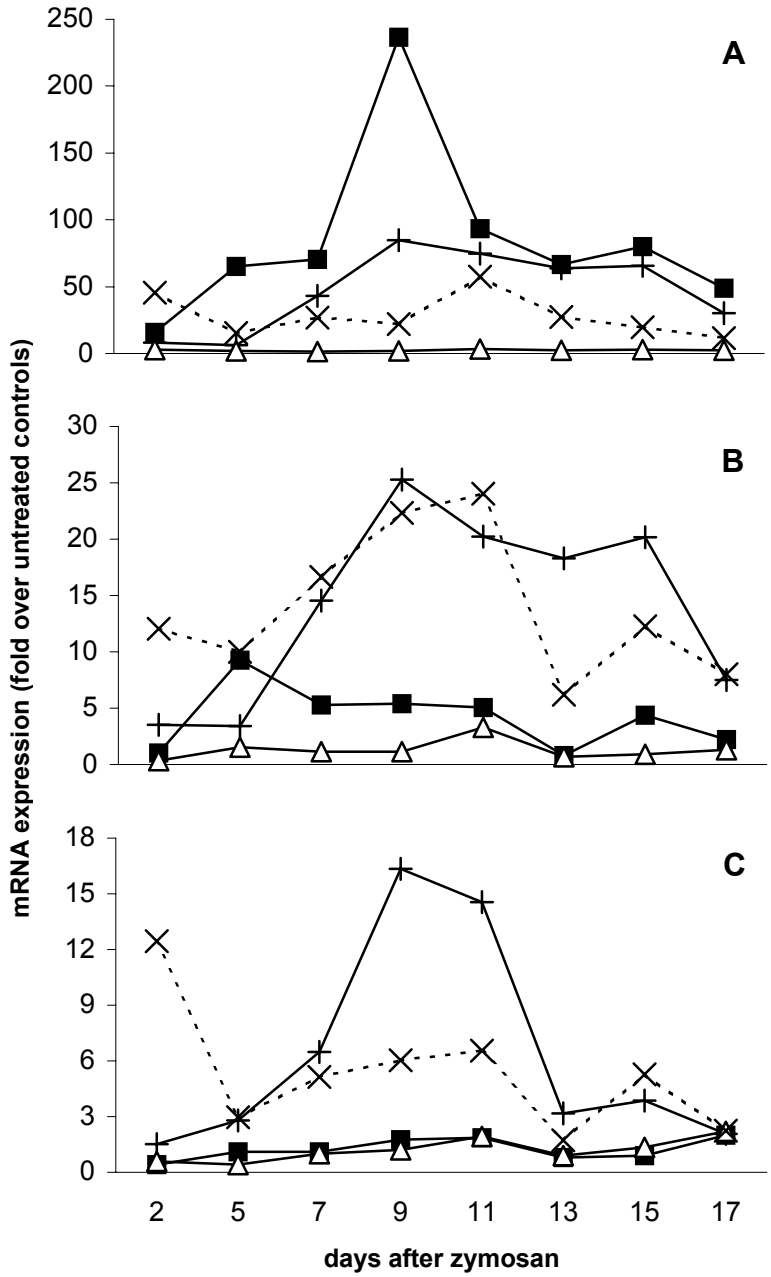

Figure 3. Upregulation of TNF-α (A), IL-1β (B), IL-6 (C) mRNA. The degree of upregulation was calculated for each animal as the mRNA expression at any time point in relation to the median mRNA expression in control mice. Data are represented as medians. +liver, ■lungs, Δspleen, ×kidneys, n=6 per organ per time point.

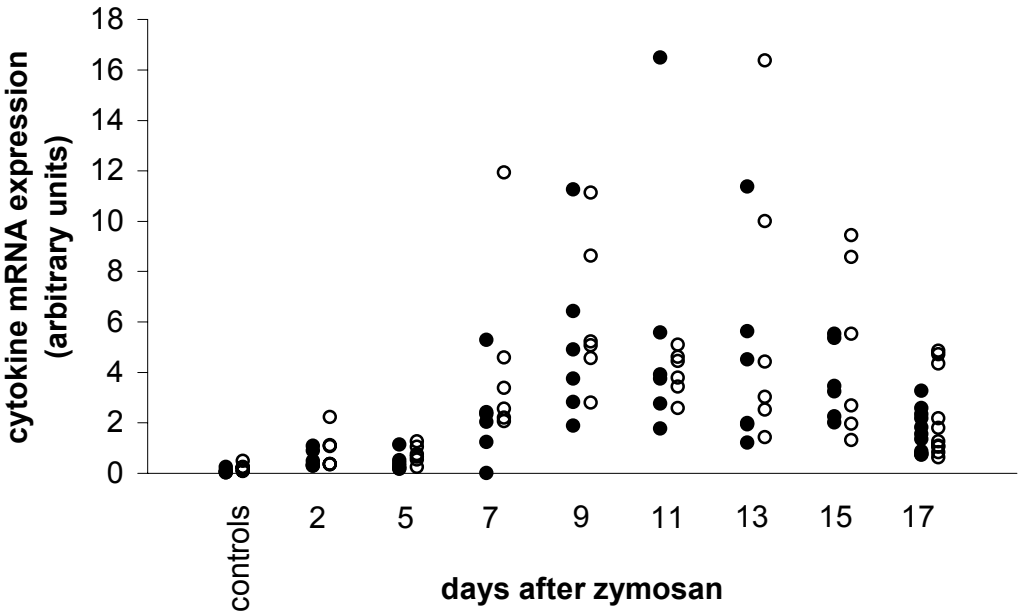

Figure 4. Cytokine mRNA expression in the liver. Individual values of mRNA for TNF-α (●) and IL-1β (○), quantitated by a real-time RT-PCR, in livers from untreated control mice and zymosan-treated animals.

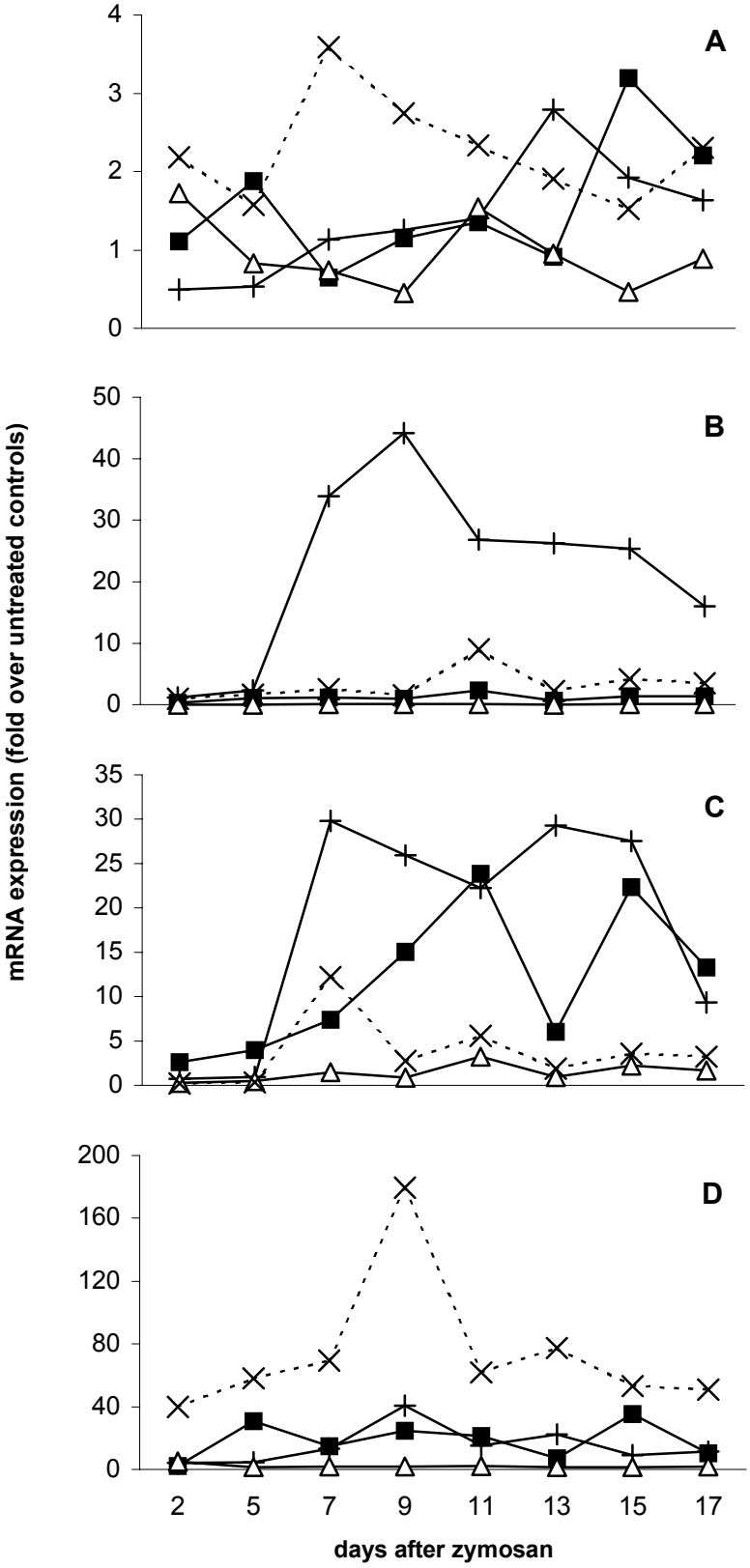

Figure 5. Upregulation of MIF (A), IL-12 (B), IFN-γ (C) and IL-10 (D) mRNA. The degree of upregulation was calculated for each animal as the mRNA expression at any time point in relation to the median mRNA expression in control mice. Data are represented as medians. +liver, ■lungs, Δspleen, ×kidneys, n=6 per organ per time point.

organs. In the early days after zymosan administration, during the acute peritonitis phase, upregulation appears to be limited. During the period of apparent recovery, cytokine mRNA expression strongly increases, mostly reaching its maximum at day 9 when deterioration of the clinical condition has already set in.

The cytokines that were examined in this study have all been related to systemic inflammation [2] and could therefore play a role in the pathophysiology of MODS. Of the cytokines examined, the upregulation of TNF-α mRNA is most pronounced, especially in the lungs and liver. The rise in TNF-α mRNA in the lungs precedes that in the liver, which agrees with the general observation that the lungs are the first organs affected during the development of MODS.

These findings, which indicate a major role for TNF-α in the pathophysiology, extend and support data previously reported. We have found elevated levels of TNF-α in the circulation during the third phase as well as a significantly enhanced lipopolysaccharide(LPS)-stimulated production of TNF-α by peritoneal cells, predominantly macrophages, over the entire experimental period [10]. Moreover, in TNF-α-lymphotoxin-α knockout (TNF/LT-/-) mice both survival and clinical condition were improved after zymosan challenge if compared to their wildtype controls [18]. Similar results have been reported by others for TNF receptor p55 knockout mice [19]. Although all these data suggest an important role for TNF-α in the development of organ damage, it is unlikely to be the only mediator responsible since TNF/LT-/- mice still develop organ damage [18]. Also, intervention studies directed at TNF-α using a specific antibody, chlorpromazine and IL-10, respectively, could mitigate but not prevent the symptoms of zymosan-induced MODS [20-22].

The results of the current study underscore a role for other cytokines. In several organs the mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-12 and IFN-γ was elevated at certain time points, although the degree of upregulation was not as high as for TNF-α. Plasma concentrations of IL-1β and

IL-6 are consistent predictors of outcome in patients with the adult respiratory distress syndrome (ARDS) [23]. Since TNF-α induces the synthesis of IL-1 and IL-6 in many cell types, the upregulation of these two cytokines in this study might be explained by the upregulation of TNF-α. However, this effect cannot occur in TNF knockout mice, and these mice still develop organ damage. Cuzzocrea et al. [24] found an improved clinical condition in a non-septic shock model using either IL-6 knockout mice or antibodies against IL-6, suggesting a major role of IL-6 in systemic inflammation. Also, concentrations of circulating IL-6 have been found to parallel disease severity in septic patients [25]. However, IL-6 is fairly well tolerated when given to dogs [26], making it unlikely that IL-6 is the driving force behind the physiology of inflammatory diseases.

IL-12 and IFN-γ are produced during sepsis and endotoxemia [27-29], and are necessary for an adequate immune response against invading microorganisms [30,31]. However, IL-12 and IFN-γ may also play a detrimental role in a systemic inflammatory response. Administration of anti-IL-12 antibodies in a murine model of endotoxemia resulted in a lower mortality [32]. Also, Lauw et al. [33] found that a single injection of IL-12 caused a delayed and sustained activation of multiple inflammatory pathways in the absence of detectable plasma concentrations of TNF and IL-1β, suggesting that these pathways are independent of these two potent proinflammatory cytokines. Since IL-12 induces IFN-γ production by T cells and natural killer cells [30], it cannot be ruled out that at least a part of the observed effects in in these studies may have contributed to IFN-γ.

Because in this study mRNAs of multiple cytokines are upregulated, blocking multiple cytokines simultaneously is likely to give the best results in intervention studies. Since the expression of TNF-α, IL-1β, IL-6 and IL-12 is nuclear factor kappa B (NFκB) mediated [34], intervention studies with NFκB

inhibitors seem an obvious way to do. Unfortunately, there is still no specific NFκB inhibitor available that can be used *in vivo*.

In this model, we did not find a high level of upregulation of MIF mRNA in any organ. The murine genome contains several pseudogenes for MIF. It is, however, very unlikely that these can confound our quantitative PCR, since transcription of MIF pseudogenes has never been observed [35]. Our finding of limited upregulation of MIF message does, however, not necessarily mean that MIF does not play an important role in the onset of MODS in mice. The expression of MIF mRNA was very high in most organs, but since MIF mRNA was constitutively expressed, this did not result in a high level of upregulation. Monocytes/macrophages have been shown to contain large quantities of preformed MIF that is released after several stimuli, such as LPS or TNF-α [36]. Therefore, MIF mRNA production might not be a good predictor of actual MIF activity.

In the liver and lungs, but especially in the kidneys we found a high level of upregulation of IL-10. This cytokine is regarded as an anti-inflammatory cytokine because of its inhibitory effect on the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α and IFN-γ [37]. The release of IL-10 by macrophages in response to LPS is regarded as a natural anti-inflammatory response mitigating cytokine production [16]. Also, IL-10 is produced in patients with circulatory shock from septic and nonseptic origin, and its production correlates positively with the intensity of the inflammatory response [38]. It is conceivable that also in this model for MODS, IL-10 is produced to mitigate the severe inflammatory response. The extremely elevated IL-10 mRNA levels observed in the kidneys could possibly provide an explanation for the fact that the organ damage in the kidneys in this model is limited compared to liver, lungs and spleen. To our knowledge, there are no related pathological conditions in which IL-10 is abundantly expressed in kidneys.

The liver was the organ in which the most cytokine mRNA upregulation could be seen. Except for MIF, all examined cytokines were upregulated more than tenfold at any time point in the liver, whereas in the lungs this was only the case for TNF-α, IFN-γ, and IL-10 and in the spleen for no cytokine at all. In the kidneys, however, all examined cytokines except MIF and IL-12 were upregulated more than tenfold at any time point. This is not completely in agreement with the occurrence of organ damage, because the damage in the lungs is the most severe, whereas the kidneys are relatively unaffected. If, however, the occurrence of organ damage is mainly mediated by TNF-α, this would provide an explanation for the severe lung damage observed in this model, since the upregulation of TNF-α was extremely high in the lungs.

The current data pertain to cytokine message and not to actual levels of cytokine protein. It is generally believed that a strongly increased presence of mRNA, such as demonstrated here, will translate into increased levels of the biologically active protein. However, as with all studies on gene expression, one should realize that a discrepancy between protein production and mRNA levels remains possible. Still, with the techniques available it is extremely difficult to quantitate tissue levels of mediator proteins such as cytokines.

The experimental procedures used in this study do not allow the identification of the cell types responsible for the increased cytokine mRNA expression in the organs. Previous studies in our laboratory [5,18] have demonstrated an influx of inflammatory cells in the various tissues during the development of organ damage. For instance, there is an influx of macrophages and neutrophils in the liver, there are accumulations of lymphocytes, plasma cells and neutrophils in the lungs, and accumulations of macrophages in the capsule of the spleen. Although it seems likely that these inflammatory cells are responsible for the increased presence of cytokine mRNA, we cannot rule out that resident cells also exhibit an increased gene expression.

The overall view that arises from the current data is that the upregulation of mRNA for a number of pro-inflammatory mediators becomes most explicit from 5 days after the zymosan challenge onwards, coinciding in time with the development of microscopic organ damage as reported before [5]. This finding is of particular interest with respect to future intevention studies. Since cytokine mRNA levels only peak after the onset of the MODS-like phase and upregulation appears limited in the early days, there seems to be ample room for interventions after the initial challenge, perhaps even extending well into the second – recovery – phase of the model. Obviously, one should be exceedingly careful to extrapolate the results to the clinical situation. Even so, if we hypothesize that the systemic inflammation, which is believed to be the driving force for the development of MODS in patients, comprises a similar pattern of cytokine mRNA upregulation, this would offer a window of opportunity for intervention well after the original insult. Next to the conclusion that most likely multiple cytokines should be targeted, this appears a promising finding which needs to be explored further.

ACKNOWLEDGMENT

We thank Louis van der Locht (Department of Hematology) for his expert technical assistance with the real-time PCR procedures.

References

1. Seely AJ, Christou NV. Multiple organ dysfunction syndrome: exploring the paradigm of complex nonlinear systems. Crit Care Med 2000; 28:2193-2200.

2. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit Care Med 2001; 29:S99-106.

3. Papathanassoglou ED, Moynihan JA, Ackerman MH. Does programmed cell death (apoptosis) play a role in the development of multiple organ dysfunction in critically ill patients? a review and a theoretical framework. Crit Care Med 2000; 28:537-549.

4. Goris RJ, Boekholtz WK, van Bebber IP et al. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

5. Jansen MJ, Hendriks T, Verhofstad AA et al. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

6. Volman TJH, Goris RJ, van der Jagt M et al. Organ damage in zymosan-induced multiple organ dysfunction syndrome in mice is not mediated by inducible nitric oxide synthase. Crit Care Med 2002; 30:1553-1559.

7. Demling R, Lalonde C, Youn YK et al. Lung oxidant changes after zymosan peritonitis: relationship between physiologic and biochemical changes. Am Rev Respir Dis 1992; 146:1272-1278.

8. Shayevitz JR, Miller C, Johnson KJ et al. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. Shock 1995; 4:389-396.

9. Cuzzocrea S, McDonald MC, Mazzon E et al. Beneficial effects of tempol, a membrane-permeable radical scavenger, on the multiple organ failure induced by zymosan in the rat. Crit Care Med 2001; 29:102-111.

10. Jansen MJ, Hendriks T, Vogels MT et al. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

11. Marshall JC, Creery D. Pre-clinical models of sepsis. Sepsis 1998; 2:187-197.

12. Roumen RM, Hendriks T, Ven-Jongekrijg J et al. Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. Ann Surg 1993; 218:769-776.

13. Borrelli E, Roux-Lombard P, Grau GE et al. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. Crit Care Med 1996; 24:392-397.

14. Panacek EA, Kaul M. IL-6 as a marker of excessive TNF-alfa activity in sepsis. Sepsis 1999; 3:65-73.

15. Geppert A, Steiner A, Zorn G et al. Multiple organ failure in patients with cardiogenic shock is associated with high plasma levels of interleukin-6. Crit Care Med 2002; 30:1987-1994.

16. Marchant A, Bruyns C, Vandenabeele P et al. Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia. Eur J Immunol 1994; 24:1167-1171.

17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.

18. Volman TJH, Hendriks T, Verhofstad AA et al. Improved survival of TNF-deficient mice during the zymosan-induced multiple organ dysfunction syndrome. Shock 2002; 17:468-472.

19. Burdon D, Tiedje T, Pfeffer K et al. The role of tumor necrosis factor in the development of multiple organ failure in a murine model. Crit Care Med 2000; 28:1962-1967.

20. Jansen MJ, Hendriks T, Hermsen R et al. A monoclonal antibody against tumour necrosis factor-alpha improves survival in experimental multiple organ dysfunction syndrome. Cytokine 1998; 10:904-910.

21. Jansen MJ, Hendriks T, Knapen MF et al. Chlorpromazine down-regulates tumor necrosis factor-alpha and attenuates experimental multiple organ dysfunction syndrome in mice. Crit Care Med 1998; 26:1244-1250.

22. Jansen MJ, Hendriks T, de Man BM et al. Interleukin 10 mitigates the development of the zymosan-induced multiple organ dysfunction syndrome in mice. Cytokine 1999; 11:713-721.

23. Meduri GU, Headley S, Kohler G et al. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. Chest 1995; 107:1062-1073.

24. Cuzzocrea S, de Sarro G, Costantino G et al. Role of interleukin-6 in a non-septic shock model induced by zymosan. Eur Cytokine Netw 1999; 10:191-203.

25. Damas P, Ledoux D, Nys M et al. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann Surg 1992; 215:356-362.

26. Preiser JC, Schmartz D, Van der Linden P et al. Interleukin-6 administration has no acute hemodynamic or hematologic effect in the dog. Cytokine 1991; 3:1-4.

27. Calandra T, Baumgartner JD, Grau GE et al. Prognostic values of tumor necrosis factor/cachectin, interleukin-1, interferon-alpha, and interferon-gamma in the serum of patients with septic shock. Swiss-Dutch J5 Immunoglobulin Study Group. J Infect Dis 1990; 161:982-987.

28. Heinzel FP, Rerko RM, Ling P et al. Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. Infect Immun 1994; 62:4244-4249.

29. Hazelzet JA, Kornelisse RF, Pouw-Kraan TC et al. Interleukin 12 levels during the initial phase of septic shock with purpura in children: relation to severity of disease. Cytokine 1997; 9:711-716.

30. Curfs JH, Meis JF, Hoogkamp Korstanje JA. A primer on cytokines: sources, receptors, effects, and inducers. Clin Microbiol Rev 1997; 10:742-780.

31. Zantl N, Uebe A, Neumann B et al. Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis. Infect Immun 1998; 66:2300-2309.

32. Wysocka M, Kubin M, Vieira LQ et al. Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. Eur J Immunol 1995; 25:672-676.

33. Lauw FN, Dekkers PE, te-Velde AA et al. Interleukin-12 induces sustained activation of multiple host inflammatory mediator systems in chimpanzees. J Infect Dis 1999; 179:646-652.

34. Sun Z, Andersson R. NF-kappaB activation and inhibition: a review. Shock 2002; 18:99-106.

35. Kozak CA, Adamson MC, Buckler CE et al. Genomic cloning of mouse MIF (macrophage inhibitory factor) and genetic mapping of the human and mouse expressed gene and nine mouse pseudogenes. Genomics 1995; 27:405-411.

36. Calandra T, Bernhagen J, Mitchell RA et al. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J Exp Med 1994; 179:1895-1902.

37. Smith EM, Cadet P, Stefano GB et al. IL-10 as a mediator in the HPA axis and brain. J Neuroimmunol 1999; 100:140-148.

38. Marchant A, Alegre ML, Hakim A et al. Clinical and biological significance of interleukin-10 plasma levels in patients with septic shock. J Clin Immunol 1995; 15:266-273.

4

Improved survival of TNF-deficient mice during the zymosan-induced multiple organ dysfunction syndrome.

Volman,TJH; Hendriks,T; Verhofstad,AAJ; Kullberg,BJ; Goris,RJA

Shock. 2002 Jun; 17(6): 468-72

ABSTRACT

The purpose of the study was to investigate the course of the zymosan-induced multiple organ dysfunction syndrome (MODS) in the absence of Tumor Necrosis Factor (TNF) in a murine model. 36 TNF-α/LT-α knockout (-/-) mice and 36 wildtype (+/+) mice received 40 μg LPS ip, followed by zymosan at a dose of 1 mg/g body weight 6 days later (day 0). Animals were monitored daily for body weight and temperature and clinical symptoms. At day 22, most of the surviving mice were killed to examine organ weight and histology. A small number of animals was followed until day 48. In all animals zymosan induced an acute sterile peritonitis phase followed by an apparent recovery. From day 8 onwards the TNF/LT+/+ mice entered a third - MODS-like - phase, characterized by loss of body weight, decreased body temperature and significant mortality. At day 22, survival in the TNF/LT-/- mice (92%) was significantly (P=0.01) higher than in the TNF/LT+/+ mice (60%). In addition, average body temperature and average relative (vs. weight at day 0) body weight were higher in the TNF/LT-/- mice than in the TNF/LT+/+ mice (35.9°C and 100% versus 33.3°C and 84%, respectively). However, at this time point, surviving animals from both groups showed similar and significant organ damage, indicated by an increase in absolute and relative (vs body weight) weight of lung, spleen and liver (liver only in the TNF/LT-/- mice). Moreover, histopathological examination of organs from the surviving animals showed a similar degree of microscopic damage in both groups. Interestingly, besides mononuclear cells, inflammatory infiltrates in lungs and livers of TNF/LT+/+ but not of TNF-/- mice contained neutrophils. In conclusion, TNF-deficient mice exhibit significantly improved morbidity and mortality during zymosan-induced MODS. However, the absence of TNF does not completely protect against MODS in this murine model.

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is characterized by serious inflammation that leads to deterioration of several organ functions, starting mostly with lung failure and followed by failure of the liver, gut and kidneys [1]. It is the principal cause of death on the intensive care unit in patients suffering from multiple trauma, shock, peritonitis, or pancreatitis [1,2].

Despite our much increased understanding of infection, sepsis, trauma and cell and organ injury over the last decades, the exact pathophysiology of MODS remains to be elucidated. The syndrome involves organs that are not necessarily directly involved in the primary disease [1]. Furthermore, between the primary disease or insult and the development of MODS lies a period of days to weeks. In the mid-1980s it was suggested that MODS might result not from infection per se, but from an uncontrolled, systemic inflammatory response [3]. This hypothesis is now generally accepted [2,4-7]. Since its initial description in 1973 [8], no substantial improvement of treatment strategy has occurred. Once MODS develops, the morbidity and mortality remain high, so prevention is still the best solution for MODS. Aggressive support of normal organ function is currently the best way to prevent MODS [9].

Research of MODS in humans is hampered by the fact that the patients at risk for MODS do not constitute a very homogenous group. Also, the examination of the mechanisms behind MODS requires invasive procedures, which cannot be easily performed on humans. In our laboratory, an animal model for MODS has been developed [10,11] and since then it has been adopted by others, sometimes in modified versions [12-14]. In this model, intraperitoneal administration of zymosan in a paraffin oil suspension leads to a three phasic illness, consisting of an acute sterile peritonitis, followed by a period of apparent recovery and, finally, the development of MODS-like symptoms, characterized by progressive damage in multiple organs [11].

Since MODS is now believed to be the result of a generalized inflammation, much attention has focused on the role of pro-inflammatory mediators in its pathophysiology [15]. TNF-α and lymphotoxin(LT)-α are pro-inflammatory cytokines and members of the TNF-family. They both bind to the same receptors, induce overlapping intracellular pathways and lead to similar biological effects [16]. MODS usually occurs in patients with sepsis, and it is well-established that TNF-activity is elevated during sepsis [17]. Previous experiments [18] in our laboratory have shown that plasma TNF-α levels were increased during the first 24 h after administration of zymosan in mice, followed by a prominent peak from day 8 onwards. Moreover, subsequent experiments revealed that interventions directed at TNF could mitigate the symptoms of zymosan-induced MODS, indicating a critical role for TNF in the pathophysiology. These interventions consisted of the administration of the anti-inflammatory cytokine IL-10 [19], a monoclonal antibody against TNF-α [20], or chlorpromazine [21]. These findings raise the question whether zymosan-induced MODS can develop in the absence of TNF. Because both TNF and LT interact with the same membrane receptors and have similar effects, the absence of one of these cytokines may be partly compensated by actions of the other one. To exclude the possible redundant effects of the two cytokines, we investigated the course of zymosan induced generalized inflammation in TNF-α/LT-α double knockout mice.

MATERIAL & METHODS

Animals

Experiments were performed with TNF-α and LT-α double knockout (TNF/LT -/-) mice and wildtype (TNF/LT+/+) mice, 7 to 9 weeks old, weighing 20-25 g. The TNF/LT-/- mice were characterized elsewhere [22]. The animals were fed standard chow (Hope Farms RMB-H, Woerden, The Netherlands) and acidified

water ad libitum. The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 5 days. The experiments were approved by the Animal Ethics Review Committee of the University Medical Centre Nijmegen, in adherence to the NIH guidelines for the use of experimental animals.

Zymosan-induced generalized inflammation

The zymosan-induced generalized inflammation (ZIGI) model for MODS has been described previously [11]. In order to improve reproducibility and to limit mortality in the first phase, a small modification to the initial model was introduced, consisting of an aseptic intraperitoneal injection of 40 µg LPS (E.coli, Sigma, St. Louis, MO) dissolved in 200 µl PBS, six days prior to zymosan administration. Zymosan A (Sigma, St. Louis, MO) was sterilized by gamma radiation (5 kGray) and homogeneously suspended in sterile paraffin oil (25 mg/ml) by high frequency vibration during 1 h. After sonification, the suspension was sterilized again in a waterbath at 100°C for 80 min. All suspensions were freshly made before use. Zymosan was given intraperitoneally in a dose of 1 mg/g body weight.

Experimental design

Two experiments were performed. In experiment 1, 24 TNF/LT-/- mice and 25 TNF/LT+/+ mice were treated with LPS and zymosan. Six additional animals (three of each group) received neither zymosan nor LPS and acted as controls. Survival, body weight and body temperature were monitored. At day 22, 10 TNF/LT-/- mice and 5 TNF/LT+/+ mice were killed by cervical dislocation and the lungs, liver, and spleen were collected and weighed. Lung damage was assessed macroscopically, using an arbitrary lung score: 0: no hemorrhages 1: hemorrhagic spots covering approximately 1-25% of the lung surface; 2:

confluent hemorrhagic spots covering approximately 25-50% of the lung surface; 3: great areas of hemorrhaging visible covering 50-75% of the lung surface; 4: completely hemorrhagic lungs (75-100% of the surface covered with hemorrhages). In order to ensure that the TNF/LT-/- would not display a high mortality later on, the surviving animals were followed until day 48, when all animals were killed.

In experiment 2, 12 TNF/LT-/- mice and 11 TNF/LT+/+ mice were treated with LPS and zymosan and again survival, body weight and body temperature were monitored. At day 22, the surviving animals were killed and the lungs, liver, and spleen were harvested for histology. The organs were fixed for 24 hours in phosphate buffered 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4µm in thickness were stained with hematoxylin-eosin.

Statistical analysis

Statistical significance of differences in mortality between experimental groups was determined using a two-sided Fisher's exact test. Statistical significance of differences in body temperature, relative body weight, relative organ weights and lung scores were determined using a two-sided Mann-Whitney U test. P-values smaller than 0.05 were considered significant.

RESULTS

Clinical condition

After the injection of LPS on day –6, the animals became ill for one day, indicated by a transient loss in body weight. By the time they received zymosan (day 0), the animals had recovered completely. If mice received LPS only and

no zymosan, their clinical course ran equal to that observed for the control group which received neither LPS nor zymosan (results not shown).
The injection of zymosan induced an acute sterile peritonitis rendering all animals very ill during the first two days, reflected by a ruffled fur, diarrhea, lethargic behavior and a decrease in body weight. The TNF/LT+/+ mice but not the TNF/LT-/- mice also displayed a transient loss of body temperature. During the second phase of the illness (day 3 to 5) the condition of the surviving animals appeared to return to normal, reflected by the display of natural curiosity, grooming, a smooth fur, and disappearance of diarrhea. Body temperature was restored and animals gained weight again. From day 6 onwards, animals became ill again, indicated by lethargic behavior, breathing difficulties, weight loss and a decrease in body temperature. Weight loss and decrease in body temperature were less severe in the TNF/LT-/- mice (Figure 1). On day 22, the average body temperature of the surviving TNF/LT+/+ mice was 33.3±3.1˚C. For TNF/LT-/- mice this was 35.9±1.0˚C (P=0.06). The average relative (vs. weight at day 0) body weight of surviving TNF/LT+/+ mice was 85±18.9% versus 100±10.2% for the TNF/LT-/- mice (P=0.01).
In the second experiment, performed for histological analysis of the organs, the clinical course of the illness was similar to the first experiment (data not shown).

Survival

The TNF/LT+/+ mice showed a mortality of 8% during the first phase after zymosan administration. Although the TNF/LT-/- mice also became ill, none died in this early phase. During the recovery phase, there was no mortality in both groups. In the third -MODS-like- phase, the survival of the TNF/LT-/- mice remained high: at day 22 it was 92%, which was significantly (P=0.02) higher than the 60% survival of the TNF/LT+/+ mice (Figure 2).

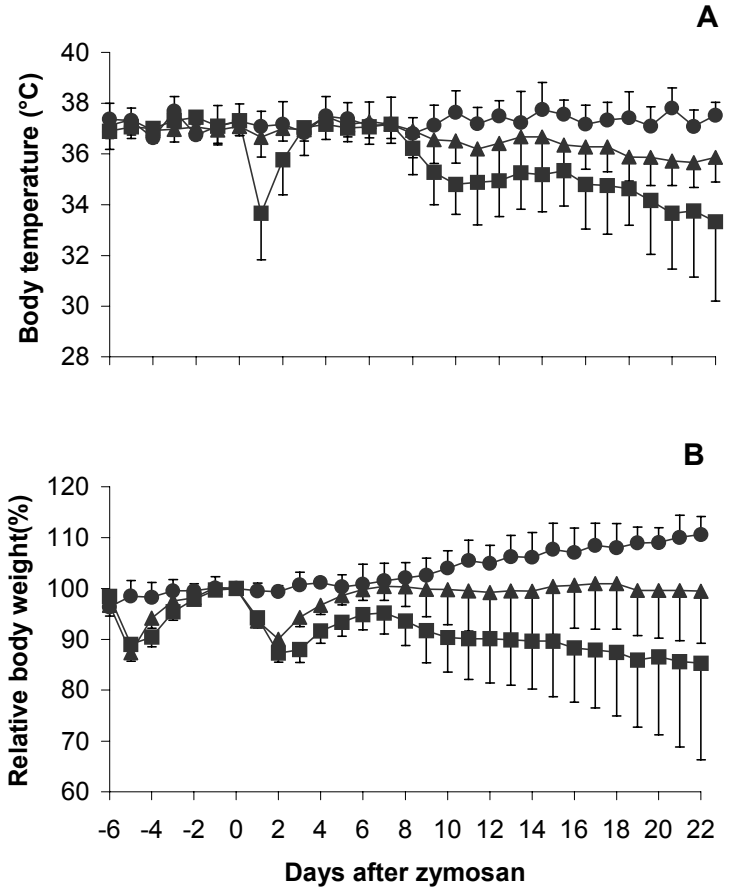

Figure 1. *Body temperature (A) and relative body weight (B) of surviving TNF/LT-/-(▲, n=22), TNF/LT+/+ (■, n=15) and control mice (●, n=6). Data are presented as mean and SD. Relative body weight was calculated as (body weight/body weight at day 0) * 100%. Relative body weight was significantly different between the TNF/LT-/- and the TNF/LT+/+ group at day 22, the difference in body temperature almost reached significance (body temperature: P=0.06, relative body weight: P=0.01 by Mann-Whitney U test).*

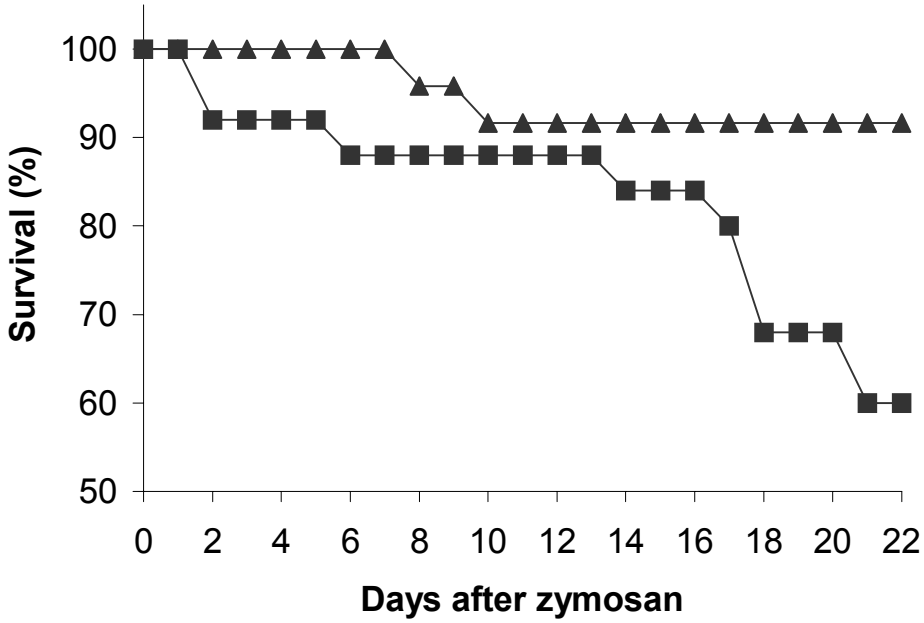

Figure 2. *Survival of TNF/LT-/- (▲, n=24 at day 0) and TNF/LT+/+ (■, n=25 at day 0) mice after administration of zymosan. At day 22, survival was significantly better in the TNF/LT-/- mice (P=0.02 by Fisher's exact test).*

12 TNF/LT-/- mice and 10 TNF/LT+/+ mice were followed until day 48, in order to rule out the possibility that the TNF/LT-/- mice would display a high mortality after day 22. This was not the case: 2 out of 12 surviving TNF/LT-/- mice died between day 22 and day 48. For the TNF/LT+/+ mice this number was 3 out of 10.
In the second experiment, performed for histological analysis of the organs, survival at day 22 was 91% in the TNF/LT-/- mice and 30% in the TNF/LT+/+ mice. (These data were not included in figure 2.)

Organ weights and lung scores

To correct for differences in body weight between animals, organ weights are expressed as a percentage of total body weight (relative organ weight, figure 3). On day 22, relative lung weight was elevated in both TNF/LT-/- (P=0.01) and TNF/LT+/+ (P=0.04) mice, as compared to untreated control animals (TNF/LT -/- mice: 2.8±0.6%, TNF/LT+/+ mice: 3.2±0.9% and controls: 0.4±0.1%). However, there was no difference between both experimental groups. The average relative liver weight was higher in the TNF/LT-/- mice (8.6±2.0%) than in the TNF/LT+/+ mice (4.2±1.0%; P=0.00) and the controls (4.6±0.2%; P=0.01). Average spleen weights of both TNF/LT-/- (2.1±0.8%) and TNF/LT+/+ (1.2±0.4%) mice were higher (P=0.01 and 0.04, respectively) than controls (0.3±0.0%). Spleen weights of TNF/LT-/- mice were even higher than those of TNF/LT+/+ mice (P=0.03).
Macroscopic lung scores were not significantly different between TNF/LT-/- and TNF/LT+/+ mice (Figure 4).

Histopathology

Twenty-two days after the injection of zymosan, surviving TNF/LT-/- and TNF/LT+/+ mice both show a similar degree of histopathological abnormalities in the lungs, the liver and the spleen, although there was variation between animals. The lungs showed hemorrhagic areas where the alveoli had disappeared to a large extent (atelectasis). In other parts the alveoli were empty but dilated. Around the bronchi and arterioles accumulations of lymphocytes and plasma cells were seen (Figure 5A and 5B). It was remarkable that that in TNF/LT+/+ mice, these accumulations also contained neutrophils, whereas in TNF/LT-/- mice almost no neutrophils could be seen. In the liver, blood vessels were surrounded by a mononuclear infiltrate. Focal accumulations of mononuclear cells were seen all over the liver. In contrast to TNF/LT-/- mice, livers of TNF/LT+/+ mice also contained accumulations of neutrophils (Figure 5C and 5D). The capsule of the spleen was thickened, due to accumulations of macrophages containing phagocytosed material. The white pulp was depleted of lymphocytes, which resulted in the disappearance of the distinct borders between white and red pulp. The red pulp contained numerous plasma cells (Figure 5E and 5F).

DISCUSSION

In the present study, TNF/LT-/- mice exhibited a higher survival rate and better clinical condition than the TNF/LT+/+ mice. The difference in survival was most pronounced in the third phase of the model, when the MODS-like symptoms occur. However, measurement of organ weights and histological examination showed that surviving TNF/LT-/- mice had a similar degree of organ damage as the surviving TNF/LT+/+ mice and were therefore not

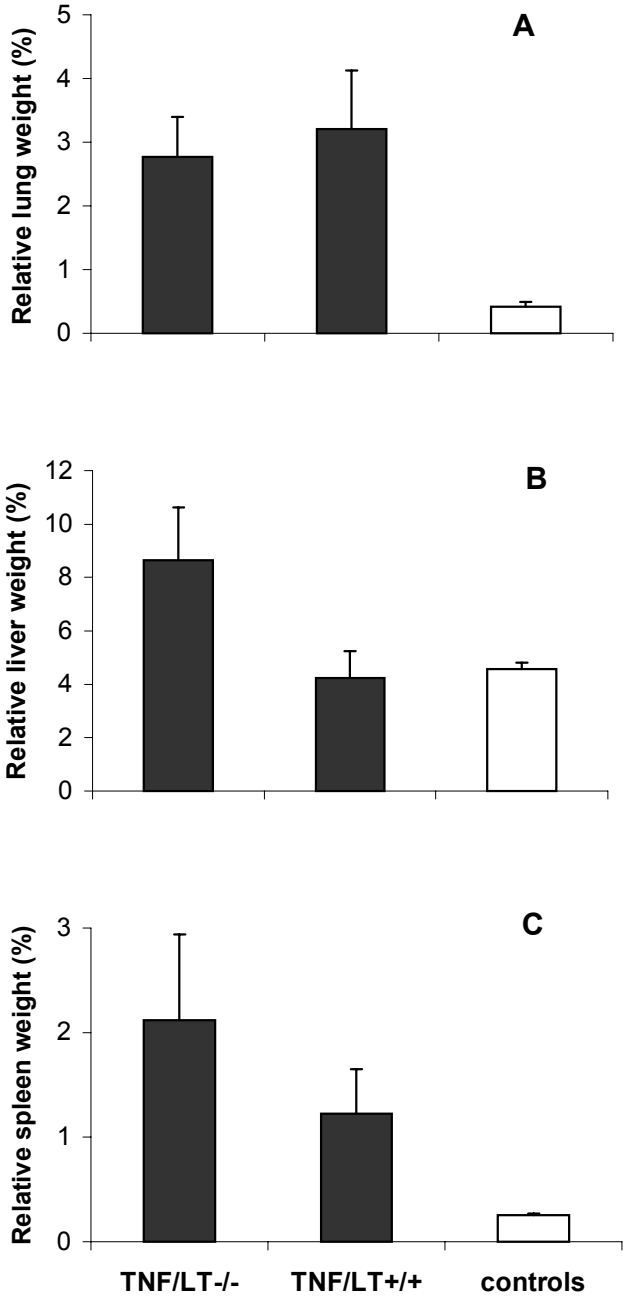

Figure 3. *Relative lung (A), liver (B) and spleen (C) weights of surviving TNF/LT-/- (n=10), TNF/LT+/+ (n=5) and control mice (n=3). Data are presented as mean and SD. Relative organ weight was calculated as: wet organ weight/body weight at day 22. Differences between TNF/LT-/- and TNF/LT+/+ mice were significant (by Mann-Whitney U test) for liver (P=0.00) and spleen (P=0.03).*

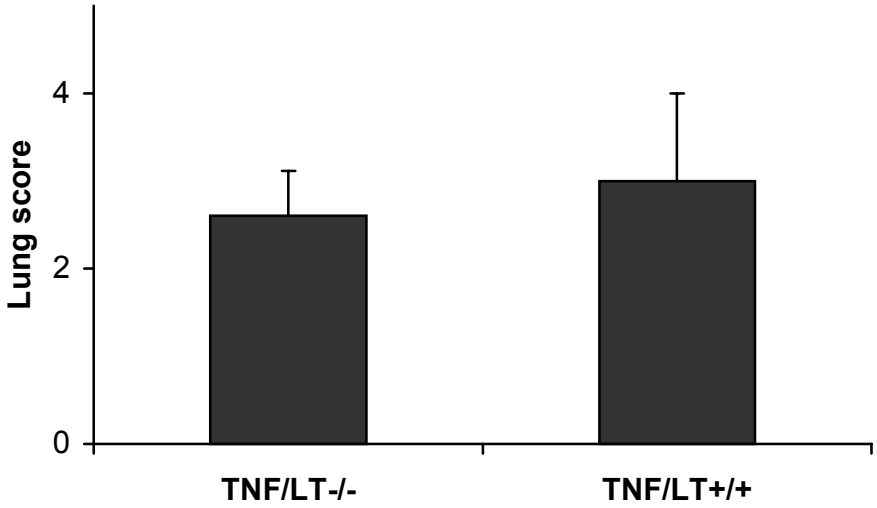

Figure 4. *Lung scores of surviving TNF/LT-/- (n=10) and TNF/LT+/+ (n=5) mice. Data are presented as mean and SD. There was no significant difference between groups (Mann-Whitney U test).*

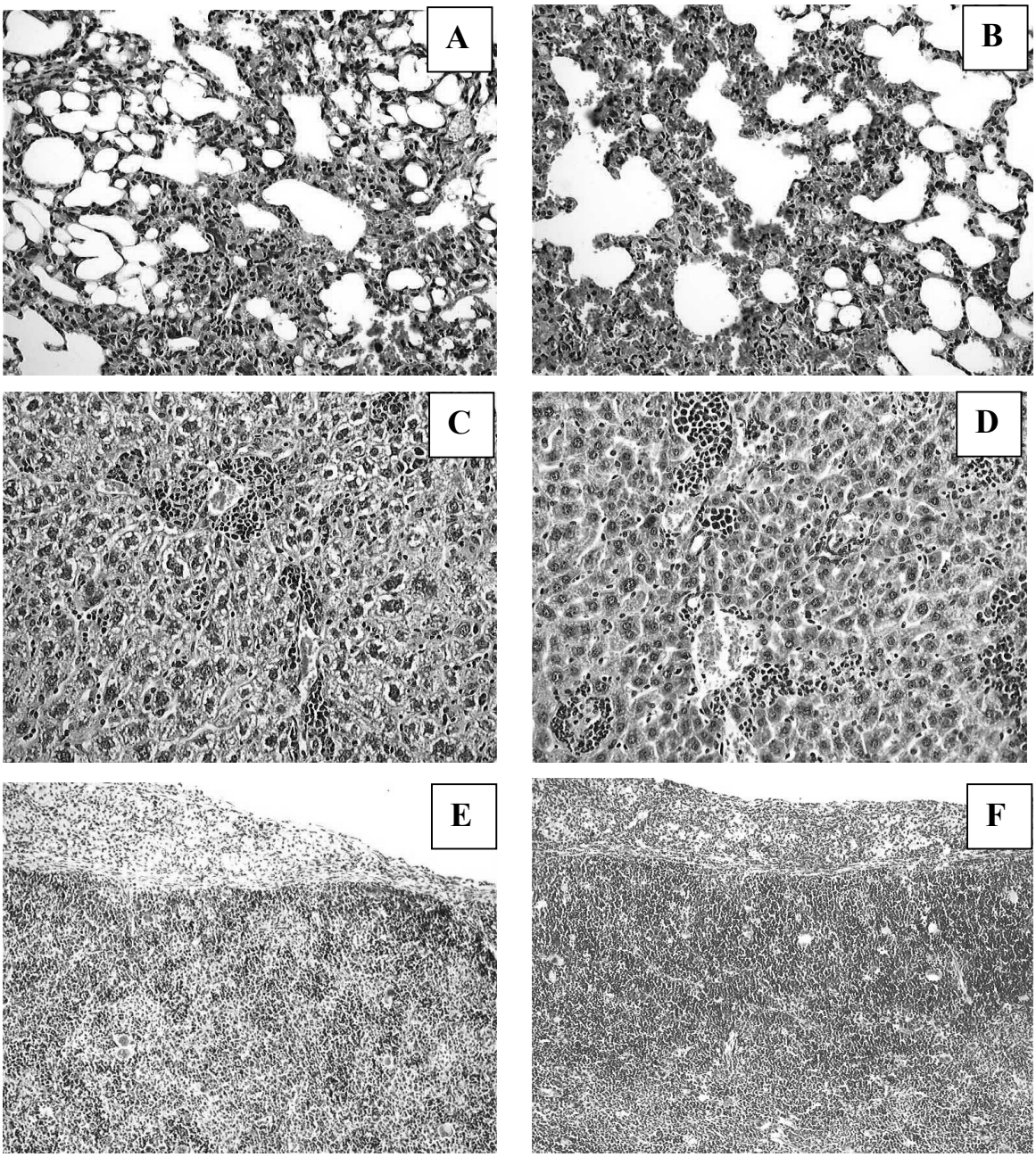

Figure 5. *Representative sections of lung (A, B), liver (C, D), and spleen tissue (E, F) from TNF/LT-/- (A, C, E) and TNF/LT+/+ (B, D, E) mice. Animals were killed 22 days after zymosan and tissue sections were stained with hematoxylin-eosin.*

protected completely against MODS. In other words, MODS can still develop in the absolute absence of TNF-α and LT-α.

It is generally accepted that TNF plays a critical role in systemic inflammation. The current observation that TNF/LT-/- mice had better survival and clinical condition than TNF/LT+/+ mice in the ZIGI model support the importance of TNF in the development of MODS. Jansen et al. [18] could show two peaks of TNF-α immunoreactivity in the serum of mice after zymosan injection. The first peak occurs in the first phase while the second peak is detectable in the third phase, when MODS-like symptoms occur in the mice. Shayevitz et al. [23] also observed elevated plasma TNF-α levels in the first phase of the model, but they give no data for the third phase. Jansen et al. [18] also found that the LPS-stimulated TNF-α production by peritoneal macrophages was strongly elevated in the second and third phase of the model. Recently published data by Burdon et al. [14] also suggest a major role for TNF in the ZIGI model. They observed an increased level of TNF-α mRNA in the livers and lungs of mice that were in the third phase. Similar results were found in our lab, although our data suggest that TNF-α mRNA in the lungs was already elevated in the second –recovery- phase of the model (unpublished results). In an experiment with TNF p55 receptor knockout mice Burdon et al. found no mortality or weight loss of these mice in the third -MODS-like- phase at all. Moreover, they found similar histopathological changes as described above in the lungs and liver of TNF-Rc-p55+/+ mice at day 14, but no changes could be observed in the lungs of the TNF-Rc-p55-/- mice, and changes in the liver could only be observed in 50% of the TNF-Rc-p55-/- mice. So, although the findings of Burdon et al. are to a large extent similar to ours, there are some differences. These differences might be due to the fact that they used a different type of knockout mice than we did (TNF-Rc-p55-/- versus TNF/LT-/-). While TNF-Rc-p55-/- possess TNF p75 receptors, Burdon at al. could not detect any TNF-α mRNA in the lungs and

livers of TNF-Rc-p55-/- mice at any time point: thus it is unlikely that the TNF p75 receptor plays a role in the development of organ damage.

Several experiments have been conducted with substances that might counteract the effects of TNF in the ZIGI model. Administration of IL-10 improved both mortality and morbidity and also reduced organ damage [13,19]. Although IL-10 is not a specific TNF inhibitor, it downregulates its production [24]. Treatment of the mice with chlorpromazine, a phenothiazine known to inhibit TNF-α production in endotoxic shock models [25], resulted in reduced organ damage, although it did not affect survival [21]. Treatment of the mice with V1q, an antibody against TNF-α resulted in a higher survival rate [20]. Thus, all these anti-TNF treatments were beneficial to some extent. However, none of the treatments could completely prevent MODS in mice.

The improvement in morbidity and mortality in TNF/LT-/- mice raises questions about the mechanisms that are responsible for the organ damage in the ZIGI model. A possible explanation for the reduced morbidity and mortality in the TNF/LT-/- mice is the observed absence of neutrophil infiltration in the lungs and livers of these mice. Impaired recruitment of neutrophils in TNF/LT-/- mice was observed earlier by Netea et al. [26] in animals that were infected with *Candida Albicans*. TNF is an important stimulus of the expression of adhesion molecules such as E-selectin and ICAM-1 [27] and a reduced expression of adhesion molecules in TNF/LT-/- mice may impair neutrophil recruitment. Apart from stimulating neutrophil recruitment, TNF is also able to strongly potentiate the function of neutrophils, resulting in superoxide production [28]. Since superoxide production may cause tissue damage [29], a reduced capacity of neutrophils from TNF/LT-/- mice to produce superoxide may be beneficial in the ZIGI model.

Since TNF/LT-/- mice still develop organ damage, it appears that, although TNF plays an important role in the pathogenesis, it can not be the only mediator responsible for MODS in mice. In the last couple of years, several mediators

have been proposed which could be important in this respect. An experiment in our laboratory with IL-1ß-/- mice showed that survival and clinical condition of these mice were not different from IL-1ß+/+ mice in the ZIGI model (unpublished observations), indicating that it is unlikely that IL-1ß plays a crucial role in the pathophysiology. Another mediator that may be important is nitric oxide (NO). Cuzzocrea et al. suggest an important role of NO in zymosan induced MODS in rats [30], but experiments in our laboratory with a selective iNOS inhibitor and with iNOS-/- mice did not confirm this (manuscript in preparation). Complement factors have also been suggested to mediate MODS in mice. Nieuwenhuijzen et al. [31], Mahesh et al. [32] and Miller et al. [33] all observed a reduced mortality in C5 deficient mice after zymosan administration, but organ damage was not prevented in these mice. IL-6 might also play a role in the development of zymosan-induced organ damage [18,34], but a recent experiment in our lab with IL-6-/- mice did not show a lower mortality of these mice (unpublished observations).

The ZIGI model is widely used to investigate the pathophysiology of MODS [12-14,23]. As opposed to animal models that are used to study sepsis, the ZIGI model is of long duration. This provides the opportunity to study the process of chronic inflammation that leads to MODS development. Because the early mortality in the ZIGI model suddenly increased dramatically in our experiments, most likely as the result of changing conditions within our central animal facility, we introduced a small modification to the ZIGI model. This modification consists of an aseptic intraperitoneal injection of 40 μg LPS six days before the intraperitoneal injection of zymosan. This way, the immune system of the animals is triggered before they receive zymosan, which prevents an excessive mortality rate in the first days after zymosan; the subsequent course of the model remains essentially unchanged. The effect of this LPS dose is limited and transient and does, in itself, not lead to any organ damage.

In conclusion, TNF-deficient mice exhibit significantly improved survival during zymosan-induced MODS. Also, the clinical condition appears to be better than in the wildtypes. Still, surviving animals from both groups eventually display a similar degree of organ damage. Thus, in this murine model, the absence of TNF does not completely protect against MODS. This implies that interventions directed only at TNF will not be sufficient in the prevention of MODS. Thus, as Arthur Baue pointed out [35], it is an illusion that we will be able to prevent MODS by a single "magic bullet". Instead, multiple agents may be necessary to modulate rather than block an excessive or continuing inflammatory response.

ACKNOWLEDGMENTS

We thank Ben de Man and Roger Lomme for their expert technical contributions.

References

1. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. Ann Surg 1992; 216:117-134.
2. Beal AL, Cerra FB. Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction. JAMA 1994; 271:226-233.
3. Goris RJ, te-Boekhorst TP, Nuytinck JK, Gimbrere JS. Multiple-organ failure. Generalized autodestructive inflammation? Arch Surg 1985; 120:1109-1115.
4. Davies MG, Hagen PO. Systemic inflammatory response syndrome. Br J Surg 1997; 84:920-935.
5. Bone RC. Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. Crit Care Med 1996; 24:163-172.
6. Yao YM, Redl H, Bahrami S, Schlag G. The inflammatory basis of trauma/shock-associated multiple organ failure. Inflamm Res 1998; 47:201-210.
7. Goris RJ. MODS/SIRS: result of an overwhelming inflammatory response? World J Surg 1996; 20:418-421.
8. Tilney NL, Bailey GL, Morgan AP. Sequential system failure after rupture of abdominal aortic aneurysms: an unsolved problem in postoperative care. Ann Surg 1973; 178:117-122.
9. Baue AE, Durham R, Faist E. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? Shock 1998; 10:79-89.
10. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.
11. Jansen MJ, Hendriks T, Verhofstad AA, Lange W, Geeraedts-LM J, Goris RJ. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

12. Steinberg S, Flynn W, Kelley K, Bitzer L, Sharma P, Gutierrez C, Baxter J, Lalka D, Sands A, van Liew J, et a. Development of a bacteria-independent model of the multiple organ failure syndrome. Arch Surg 1989; 124:1390-1395.

13. Ferrer TJ, Webb JW, Wallace BH, Bridges CD, Palmer HE, Robertson RD, Cone JB. Interleukin-10 reduces morbidity and mortality in murine multiple organ dysfunction syndrome (MODS). J Surg Res 1998; 77:157-164.

14. Burdon D, Tiedje T, Pfeffer K, Vollmer E, Zabel P. The role of tumor necrosis factor in the development of multiple organ failure in a murine model. Crit Care Med 2000; 28:1962-1967.

15. Christman JW, Lancaster LH, Blackwell TS. Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy [see comments]. Intensive Care Med 1998; 24:1131-1138.

16. Bemelmans MH, van Tits LJ, Buurman WA. Tumor necrosis factor: function, release and clearance. Crit Rev Immunol 1996; 16:1-11.

17. van der PT, Lowry SF. Tumor necrosis factor in sepsis: mediator of multiple organ failure or essential part of host defense? [editorial]. Shock 1995; 3:1-12.

18. Jansen MJ, Hendriks T, Vogels MT, Van der Meer JW, Goris RJ. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

19. Jansen MJ, Hendriks T, de Man BM, Van der Meer JW, Goris RJ. Interleukin 10 mitigates the development of the zymosan-induced multiple organ dysfunction syndrome in mice. Cytokine 1999; 11:713-721.

20. Jansen MJ, Hendriks T, Hermsen R, Van der Meer JW, Goris RJ. A monoclonal antibody against tumour necrosis factor-alpha improves survival in experimental multiple organ dysfunction syndrome. Cytokine 1998; 10:904-910.

21. Jansen MJ, Hendriks T, Knapen MF, van Kempen LC, Van der Meer JW, Goris RJ. Chlorpromazine down-regulates tumor necrosis factor-alpha and attenuates experimental multiple organ dysfunction syndrome in mice. Crit Care Med 1998; 26:1244-1250.

22. Amiot F, Boussadia O, Cases S, Fitting C, Lebastard M, Cavaillon JM, Milon G, Dautry F. Mice heterozygous for a deletion of the tumor necrosis factor-alpha and lymphotoxin-alpha genes: biological importance of a nonlinear response of tumor necrosis factor-alpha to gene dosage. Eur J Immunol 1997; 27:1035-1042.

23. Shayevitz JR, Miller C, Johnson KJ, Rodriguez JL. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. Shock 1995; 4:389-396.

24. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. J Immunol 1994; 153:811-816.

25. Gadina M, Bertini R, Mengozzi M, Zandalasini M, Mantovani A, Ghezzi P. Protective effect of chlorpromazine on endotoxin toxicity and TNF production in glucocorticoid-sensitive and glucocorticoid-resistant models of endotoxic shock. J Exp Med 1991; 173:1305-1310.

26. Netea MG, van Tits LJ, Curfs JH, Amiot F, Meis JF, Van der Meer JW, Kullberg BJ. Increased susceptibility of TNF-alpha lymphotoxin-alpha double knockout mice to systemic candidiasis through impaired recruitment of neutrophils and phagocytosis of Candida albicans. J Immunol 1999; 163:1498-1505.

27. Ward PA. Recruitment of inflammatory cells into lung: roles of cytokines, adhesion molecules, and complement. J Lab Clin Med 1997; 129:400-404.

28. Ferrante A. Tumor necrosis factor alpha potentiates neutrophil antimicrobial activity: increased fungicidal activity against Torulopsis glabrata and Candida albicans and associated increases in oxygen radical production and lysosomal enzyme release. Infect Immun 1989; 57:2115-2122.

29. Fujishima S, Aikawa N. Neutrophil-mediated tissue injury and its modulation. Intensive Care Med 1995; 21:277-285.

30. Cuzzocrea S, Zingarelli B, Sautebin L, Rizzo A, Crisafulli C, Campo GM, Costantino G, Calapai G, Nava F, Di Rosa M, Caputi AP. Multiple organ failure following zymosan-induced peritonitis is mediated by nitric oxide. Shock 1997; 8:268-275.

31. Nieuwenhuijzen GA, Meyer MP, Hendriks T, Goris RJ. Deficiency of complement factor C5 reduces early mortality but does not prevent organ damage in an animal model of multiple organ dysfunction syndrome. Crit Care Med 1995; 23:1686-1693.

32. Mahesh J, Daly J, Cheadle WG, Kotwal GJ. Elucidation of the early events contributing to zymosan-induced multiple organ dysfunction syndrome using MIP-1alpha, C3 knockout, and C5-deficient mice. Shock 1999; 12:340-349.

33. Miller CG, Cook DN, Kotwal GJ. Two chemotactic factors, C5a and MIP-1alpha, dramatically alter the mortality from zymosan-induced multiple organ dysfunction syndrome (MODS): C5a contributes to MODS while MIP-1alpha has a protective role. Mol Immunol 1996; 33:1135-1137.

34. Cuzzocrea S, de Sarro G, Costantino G, Mazzon E, Laura R, Ciriaco E, de Sarro A, Caputi AP. Role of interleukin-6 in a non-septic shock model induced by zymosan. Eur Cytokine Netw 1999; 10:191-203.

35. Baue AE. Multiple organ failure, multiple organ dysfunction syndrome, and systemic inflammatory response syndrome. Why no magic bullets?.Arch Surg 1997; 132:703-707.

5

Pentoxifylline does not improve outcome in a murine model for the multiple organ dysfunction syndrome.

Volman,TJH; Goris,RJA; Hendriks,T

Intensive-Care-Med 2005; in press

ABSTRACT

Objective: To investigate the effects of pentoxifylline (PTX) administration in a murine model for the multiple organ dysfunction syndrome (MODS).
Design: Prospective double-blind randomized animal study
Setting: University research laboratory
Interventions and measurements: Sixty C57BL/6 mice were given an aseptic intraperitoneal injection of LPS, followed after six days by zymosan (day 0) in a dose of either 0.9 or 1.0 mg/g body weight. Starting at day 0, mice were administered PTX in a dose of 80 mg/kg body weight or saline per os every 8 h. At day 17, surviving animals were killed and their liver, lungs, spleen and kidneys were collected.
Results: Mortality, course of body temperature, body weight and macroscopic lung damage were similar between zymosan-treated groups. Administration of PTX did not result in a significantly improved survival, body temperature, body weight or macroscopic lung damage. In addition, there were no significant differences in organ weights between mice that received PTX and mice that received PBS. Although PTX inhibited the LPS-induced increase in TNF-α and IL-6 expression (but not the IL-1β expression) at both mRNA and protein level in a murine macrophage cell line, TNF-α mRNA expression in the livers of PTX-treated mice was not significantly inhibited.
Conclusions: The results reported here do not support the hypothesis that PTX improves outcome in zymosan-induced multiple organ dysfunction in mice.

INTRODUCTION

Over the past decades, advances in the support of vital organ functions have transformed acute life-threatening illness from a rapidly lethal event to a chronic state that is potentially survivable. Unfortunately, the development of intensive care medicine has created a phenomenon called the multiple organ dysfunction syndrome (MODS). MODS is a cumulative sequence of progressive deterioration of organ systems. It usually starts with respiratory trouble, leading to the acute respiratory distress syndrome (ARDS) and is followed by dysfunction of the liver, gut and kidneys. Remarkably, these organs are not necessarily involved in the primary disease [1,2]. MODS may evolve after a variety of insults like, for instance, major trauma or severe bacterial infection. It is generally believed to be the result of an overwhelming host response resulting in systemic inflammation [1,3,4]. Once MODS develops, morbidity and mortality rates are high. MODS is the leading cause of death for patients admitted to an ICU. Elucidation of the mechanisms and mediators involved is necessary to develop adequate treatment protocols. This type of research requires invasive procedures and collection of tissue samples which are virtually impossible to perform on patients. Therefore, we have developed an animal model for MODS [3,5,6], which is used widely to study systemic inflammation in relation to organ failure [7-9]. After a single insult in the form of an intraperitoneal challenge with zymosan rodents suffer an acute, peritonitis-like phase that is followed by an apparent recovery. Thereafter, a gradual deterioration occurs, characterized by increasing organ damage and dysfunction [5]. Recently, a minor modification has been introduced: 'priming' of the mice with a small amount of LPS, which in itself does not cause any organ damage at all, 6 days prior to zymosan improves the reproducibility of the model, especially with respect to the mortality in the acute phase, while subsequent development of MODS remains unaltered [6].

Previous measurements, in the circulation, on isolated peritoneal cells [10], and at organ level [11] have suggested an important role in this process for several pro-inflammatory cytokines, especially tumor necrosis factor-α (TNF-α). However, since strategies directed at counteracting TNF-α have only been partially successful [12-14], targeting multiple cytokines at the same time appears to be necessary.

Pentoxifylline (PTX) is a methylxanthine derivative that has been used for treatment of chronic occlusive arterial disease because of its rheological actions [15]. PTX acts primarily by increasing red blood cell deformability, by reducing blood viscosity and by decreasing the potential for platelet aggregation and thrombus formation [16]. In addition, PTX has effects on the inflammatory response.

It has been shown to improve survival in sepsis models by decreasing TNF-α, interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels [17,18] and to inhibit the lipopolysaccharide(LPS)-stimulated production of multiple cytokines in alveolar macrophages [19]. PTX is known to inhibit phosphodiesterase, thereby increasing cytoplasmic cyclic adenosine monophosphate levels and resulting in serious inhibition of TNF-α gene transcription [20]. Next to inhibiting cytokines, PTX is also known to reduce the TNF-α-induced upregulation of E-selectin in endothelial cells [21], which inhibits the adhesion of neutrophils. It also inhibits adhesiveness, degranulation and superoxide production of activated neutrophils [22,23] and the production of nitric oxide by macrophages [24].

Because of its ability to counteract multiple pro-inflammatory mediators simultaneously, PTX would seem a potentially useful agent to attenuate the generalized inflammatory response leading to the organ failure observed in MODS. In the present study we investigated the effects of PTX administration in the murine model for MODS.

MATERIAL AND METHODS

In vitro experiments

Murine macrophage-like cells from the J774.A1 cell line were grown to confluence in Dubecco's Modified Eagle's Medium and pre-incubated in sixfold with pentoxifylline (0.1 mg/ml and 1 mg/ml) 1 h prior to the addition of LPS (1 μg/ml). In experiment 1, cells were harvested for RNA extraction and quantification (see below) 6 h after the addition of LPS. In experiment 2, the culture supernatants were collected 24 h after stimulation with LPS. TNF-α concentrations in the supernatants were measured with specific radio immuno assay (RIA) as described previously [10]. IL-6 concentrations in the supernatants were measured with Enzyme Linked Immuno Sorbent Assay (ELISA) according to the manufacturer's instructions (Biosource, Etten-Leur, the Netherlands).

Animals

The experiment was performed with 63 C57BL/6 mice, 7 to 9 weeks old and weighing 20-25 g. The animals were fed standard chow (Hope Farms RMB-H, Woerden, The Netherlands) and acidified water ad libitum. The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 5 days. The principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and the experiment was approved by the Animal Ethics Review Committee of the Radboud University Nijmegen.

Zymosan-induced generalized inflammation

The zymosan-induced generalized inflammation model for MODS has been described extensively previously [3,5,6,10,25]. Briefly, mice were given an aseptic

intraperitoneal injection of 40 µg LPS (*Escherichia coli*, Sigma Chemical, St Louis, MO) dissolved in 200 µl of phosphate buffered saline (PBS), followed after six days by zymosan (day 0). Zymosan A (Sigma Chemical) was sterilized by gamma radiation (5 kGray) and homogeneously suspended in sterile paraffin oil (25 mg/ml) by high frequency vibration during 1 h. After sonification, the suspension was sterilized again at 100°C for 80 min. All suspensions were freshly made before use. Zymosan was given intraperitoneally in a dose of either 0.9 or 1.0 mg/g body weight.

Experimental design

Animals were randomly assigned to 5 groups. Group 1 and 2 (15 mice each) received zymosan in a dose of 0.9 mg/g body weight, groups 3 and 4 (15 mice each) received zymosan in a dose of 1.0 mg/g body weight. Group 5 (6 mice) functioned as untreated control group. Survival, body temperature and body weight were monitored daily. Starting at day 0, mice were administered PTX in a dose of 80 mg/kg body weight (groups 1 and 3) or PBS (groups 2 and 4) every 8 h. PTX powder (Sigma Chemical, St. Louis, MO) was dissolved in PBS and made freshly every day. Both PTX and PBS were administered per os in a volume of 200 µl. At day 17, surviving animals were killed and their liver, lungs, spleen and kidneys were collected and weighed. Lung damage was assessed macroscopically, using an arbitrary lung score: 0: no hemorrhages 1: hemorrhagic spots covering approximately 1-25% of the lung surface; 2: confluent hemorrhagic spots covering approximately 25-50% of the lung surface; 3: great areas of hemorrhaging covering 50-75% of the lung surface; 4: completely hemorrhagic lungs (75-100% of the surface covered with hemorrhages). The livers and lungs were snap-frozen in liquid nitrogen and stored at –80°C for subsequent mRNA extraction.

Quantitative Reverse Transciptase Polymerase Chain Reaction

Frozen livers and lungs were homogenized. Total RNA from cultured murine macrophages, livers and lungs was extracted with the guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacci [26]. RNA concentration was determined by spectrophotometric measurements with a GeneQuant II RNA/DNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of precipitated total RNA was ascertained by 1% agarose gel electrophoresis. Isolated RNA was used as a template for the production of first strand cDNA using random hexamers. Relative quantification of TNF-α, IL-1β and IL-6 mRNA was performed with a TaqMan real-time RT-polymerase chain reaction (PCR) assay on a ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Quantification of the amount of TNF-α, IL-1β and IL-6 in unknown samples was accomplished using a standard curve to determine the starting concentration of target. The standard curves were generated using RNA from a mouse macrophage cell line (J774.A1) that was stimulated with LPS (1 μg/ml) for 6 h (TNF-α and IL-1β) or 4 h (IL-6). In order to correct for variations in cDNA concentrations between samples, cytokine gene expression was normalized by dividing the starting concentration of target by the starting concentration of constitutively expressed 18S ribosomal RNA (endogenous control). Oligonucleotide primers and TaqMan probe were designed with use of the Primer Express 1.0 software (Applied Biosystems). The sequence (5'-3') of forward primer, reverse primer, and probe were CCCAGACCCTCACACTCAGATC, CCTCCACTTGGTTTGCT and CGAGTGACAAGCCTGTAGCCCACGT respectively, for TNF-α, GGACAGAATATCAACCAACAAGTGAT, ATTACACAGGACAGGTATAGATTCTTTCCT and AACGACAAAATACCTGTGGCCTTGGGC, respectively, for IL-1β and TGTATGAACAACGATGATGCACTT,

GGTACTCCAGAAGACCAGAGGAAAT and AACGACAAAATACCTGTGGCCTTGGGC respectively, for IL-6. The probe for IL-6 was a Taqman Minor Groove Binder (MGB) probe. The PCR conditions were as follows: for amplification of the cytokine product, 25 ng of cDNA was added to the PCR mixture consisting of 25 µl TaqMan Mastermix (Applied Biosystems), 12.5 pmol fluorescent probe (Applied Biosystems) and 15 pmol of each primer in a final volume of 50 µl. For amplification of the 18S rRNA product, the PCR mixture consisted of 25 µl TaqMan Mastermix and 2.5 µl of pre-developed TaqMan 18S rRNA control kit (Applied Biosystems). Again, the final volume was 50 µl. Thermal cycling conditions were 2 min at 50°C and 10 min of initial denaturation at 95°C to activate *Taq* Gold polymerase, followed by 40 cycles of 2-step PCR consisting of 15 s at 95°C and 1 min at 60°C.

Statistics

Kruskal-Wallis tests were used to test whether variation among experimental groups was greater than expected by chance. When significant, Dunn's multiple comparisons tests were performed to assess statistical significance between individual groups.

RESULTS

In vitro experiments

PTX inhibited the LPS-induced TNF-α and IL-6 production in murine macrophage-like cells in a dose-dependent manner, both at mRNA and protein level. In contrast, this was not the case for IL-1β mRNA, which expression

seemed to be increased rather than decreased by PTX (Figure 1). For instance, at the highest dose used, PTX suppressed TNF-α mRNA and protein levels by 95 and 77 %, respectively and increased IL-1β mRNA levels by 29 %.

In vivo experiments

The *in vivo* effect of PTX was examined in two separate groups of animals, receiving zymosan in doses of 0.9 and 1.0 mg/g body weight, respectively. In previous, unpublished, experiments in our laboratory the higher dose led to a slightly more pronounced degree of illness. However, in the current experiment the clinical parameters were not significantly different between groups receiving 0.9 or 1.0 mg/g zymosan. Therefore, the data from both groups were combined to make up figures 2 and 3, which represent the primary outcome parameters. After the injection of LPS on day –6, all animals became ill for one day, indicated by a transient loss in body weight. By the time they received zymosan (day 0), the animals had recovered completely. The injection of zymosan induced an acute peritonitis rendering all animals very ill during the first two days, as reflected by a ruffled fur, diarrhea, lethargic behavior and a decrease in body weight and temperature. During the second phase of the illness (day 3 to 5) the condition of the surviving animals appeared to return to normal, reflected by the display of natural curiosity, grooming, a smooth fur, and disappearance of diarrhea. Body temperature was restored and animals gained weight again. From approximately day 6 onwards, animals entered the third - MODS-like - phase, indicated by lethargic behavior, breathing difficulties, weight loss and a decrease in body temperature.

Administration of PTX did not result in significantly improved survival, body temperature, body weight or lung score (Figure 2). Organ weight was significantly higher in lung and spleen of zymosan-treated animals than in

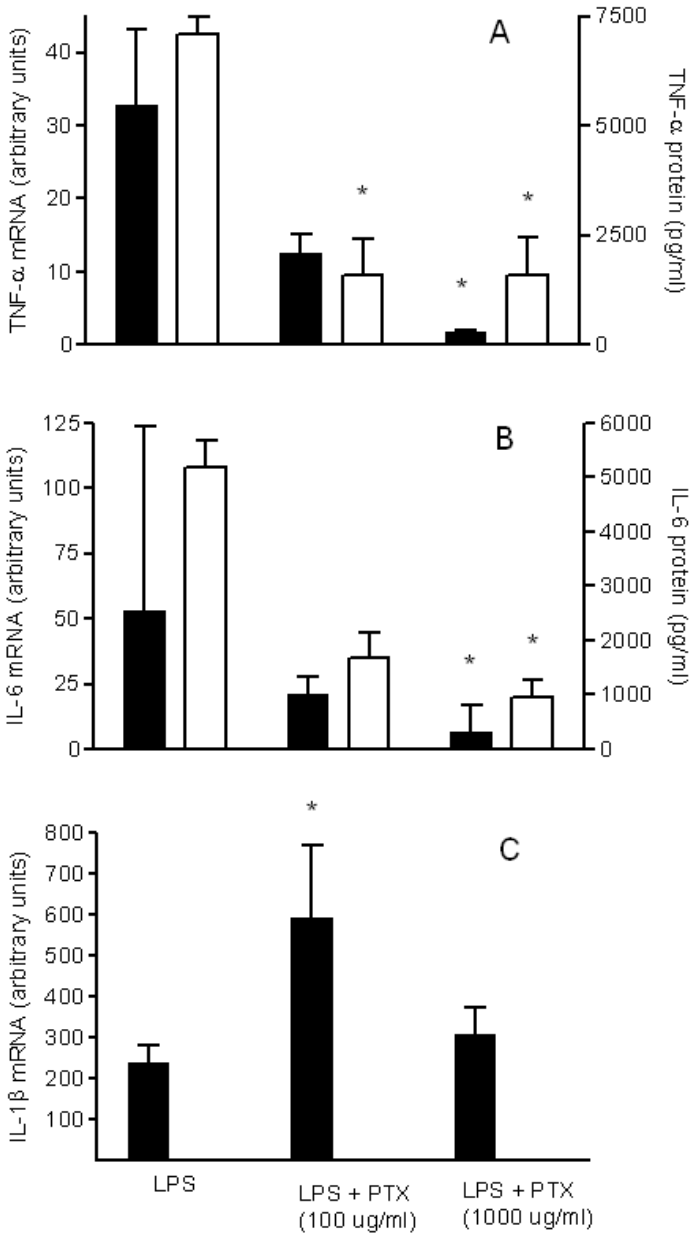

Figure 1. Effect of PTX on LPS-induced cytokine production in J774.A1 cells. Average values (+SD) for mRNA (black bars) and protein (white bars) concentration of TNF-α (A), IL-6 (B) and IL-1β (C). Samples were measured in sixfold. LPS concentration was 1 μg/ml. Asterisks denote significant differences from cells stimulated with LPS alone (Kruskal-Wallis test, if significant followed by Dunn's multiple comparison test, $P<0.05$).

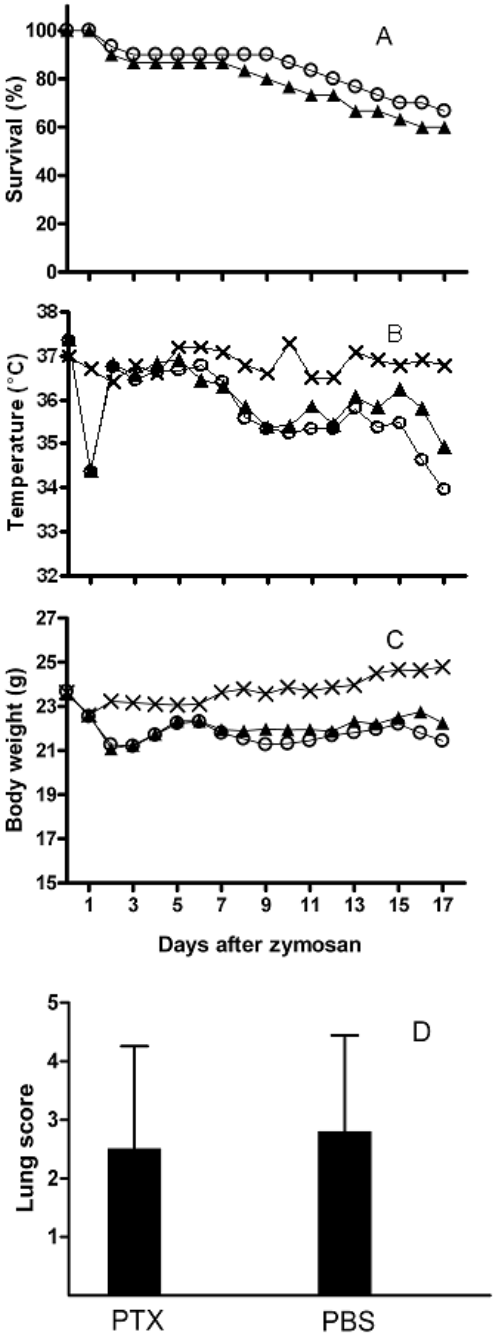

Figure 2. Effect of PTX administration on survival, clinical symptoms and lung scores. A: Survival of mice that received PTX (▲, n=30) in a dose of 80 mg/kg or PBS (○, n=30) every 8 h. B: Average body temperature and average body weight (C) of untreated control mice (x, n=6) and surviving zymosan-treated mice that were treated with PTX (▲, n=18) or PBS (○, n=20). D: Average (+ SD) lung scores of surviving mice that were treated with PTX (n=18) or PBS (n=20).

untreated controls (Figure 3). However, this parameter of organ damage was unchanged between mice that received PTX and mice that received PBS. Also, TNF-α and IL-1β mRNA expression in the liver, which was strongly and significantly increased after zymosan administration, was not significantly inhibited in PTX-treated mice (Figure 4).

DISCUSSION

Administration of PTX did not have beneficial effects on clinical condition and mortality in zymosan-induced inflammation. Upregulation of TNF-α and IL-1β mRNA in the liver was not prevented by PTX. Damage of the liver, spleen and kidneys has only been assessed indirectly by measuring organ weight since it has been demonstrated previously to correlates well with the development of histopathological damage [27]. We did investigate the course of disease after administration of both 0.9 and 1.0 mg zymosan/g body weight since, depending on the batch of zymosan, the clinical course and mortality may change rather strongly with dose (unpublished observations). However, this was not the case in the present experiment and in both series, essentially representing separate and independent experiments, PTX administration remained uneffective.
A previous study in our laboratory revealed that during zymosan-induced MODS, the mRNA expression of multiple cytokines is strongly increased [11]. The *in vitro* experiments in the current study show that PTX inhibited the LPS-induced TNF-α mRNA expression and protein production by macrophage-like cells, as was described previously in primary macrophages [19,28]. The IL-6 production was also inhibited by PTX, but this was not the case for the IL-1β mRNA expression. Earlier studies on the effects of PTX on the LPS-induced IL-1β and IL-6 production by primary macrophages have yielded conflicting results [19,28-31]. Possibly, these differences might be caused by variations in underlying disease in the species from which the macrophages were harvested.

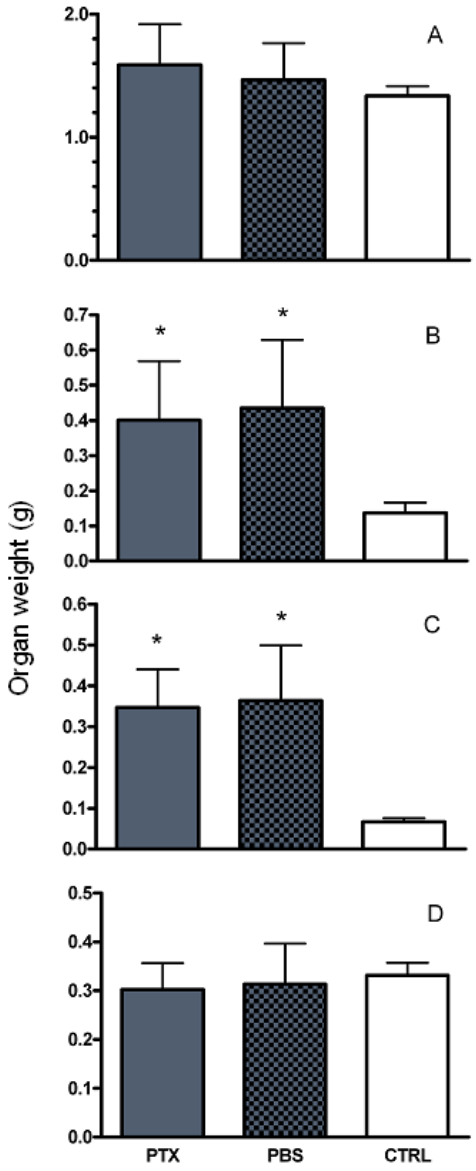

Figure 3. Effect of PTX administration on organ weight. Average values (+SD) for liver (A), lungs (B), spleen (C) and kidneys (D) in surviving zymosan-treated mice that received PTX (n=18), PBS (n=20) and in untreated control mice (n=6). Asterisks denote significant differences with respect to untreated controls (Kruskal-Wallis test, if significant followed by Dunn's multiple comparison test, P<0.05).

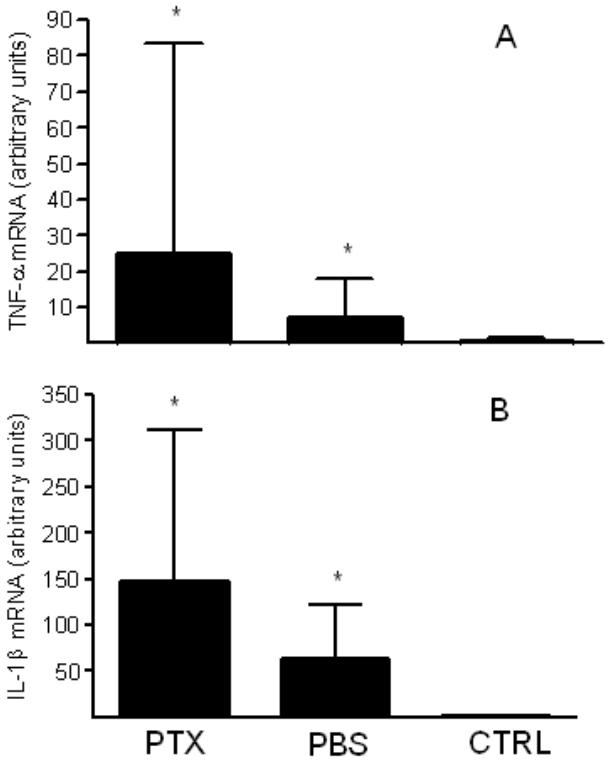

Figure 4. TNF-α and IL-1β mRNA expression in liver tissue. Average values (+SD) for surviving mice treated with zymosan in a dose of 0.9 mg/g body weight (black bars, n=11 and 10, respectively) and for untreated control mice (white bar, n=6). Mice in the experimental groups were treated with either PTX or PBS. Asterisks denote significant differences with respect to untreated controls (Kruskal-Wallis test, if significant followed by Dunn's multiple comparison test, $P<0.05$).

In the present study, no effect of PTX on TNF-α and IL-1β mRNA expression in the liver was seen *in vivo* and this is probably the main reason why no beneficial effects of PTX on morbidity and mortality were observed either. It cannot be ruled out that in other tissues than the liver mRNA levels of these cytokines were reduced, but since earlier experiments in our laboratory showed that TNF-α and IL-1β mRNA levels are most strongly upregulated in the liver during the MODS-like phase of the animal model [11], a potential reduction in mRNA levels is most likely to be detected in the liver.

A potential problem using PTX is its short half-life. Pharmacokinetic studies have shown that intraperitoneally administered PTX has a half-life in the circulation of only 4 to 7 minutes in healthy mice [32,33]. PTX is rapidly hydrolized to 1-(5-hydroxyhexyl)-3-7-dimethylxanthine, which is suggested to exhibit effects similar to those observed for PTX [34]. However, this metabolite is also eliminated rapidly [32 33]. To reduce stress for the animals and to achieve a more gradual uptake of PTX in the circulation and in the tissues, PTX was administered orally every 8 h (instead of intraperitoneally) in the present study. PTX is extensively and rapidly absorbed from the gastrointestinal tract, both in animals and in humans [16].

The results from the present study are in contrast with previous *in vivo* studies in which PTX was administered repeatedly. Kremsner et al. [35] found that daily intraperitoneal injections of 1 mg PTX abolished serum TNF bioactivity in *Plasmodium berghei*-infected mice, thereby preventing signs of cerebral malaria. Edwards et al.[36] administered IL-2, which is an antineoplastic agent, to mice with pulmonary metastases and found that intraperitoneal injections of PTX in a dose of 50 mg/kg every 12 h suppressed the rise in serum TNF levels that normally follows IL-2 administration. This way, IL-2 toxicity was limited without affecting antitumor efficacy. Louie et al. [37] reported that intraperitoneal injections of PTX in a dose of 20 mg/kg every 8 h shortened mean survival in mice with systemic *Candida albicans* infection, due to an increased organ fungal

burden. However, serum TNF-α and IL-6 levels were not decreased by PTX. In a murine colitis model [38], intraperitoneal injections of PTX (5mg/kg) twice daily significantly reduced the severity of colonic histopathology and myeloperoxidase levels. However, TNF-α could not be detected in serum of any treatment group. Koo et al. [18] administered PTX intraveneously in a dose of 50 mg/kg during a 90 minute infusion period to rats that were subjected to polymicrobial sepsis and found that this treatment attenuated the rise in plasma TNF-α, IL-1β, and IL-6 levels.

Taken together, these studies emphasize the potential beneficial effects of PTX on clinical parameters in various pathologies. In some cases, reduced circulating levels of TNF-α, IL-1β and IL-6 were reported. However, circulating cytokine levels might not reflect their activity at organ level. In the current study, we measured cytokine mRNA in liver tissue, which might be expected to correlate better with actual cytokine activity at organ level. The PTX doses used in the studies mentioned above varied between 10 and 200 mg/kg/day and were therefore lower than that employed in the current study (240 mg/kg/day). In these studies, however, PTX was administered intraperitoneally or intravenously instead of per os. In previous studies in our laboratory (unpublished data), PTX was also administered intraperitoneally every 8 h in a dose of 80 mg/kg. Here, no beneficial effect of PTX on mortality, morbidity or cytokine levels was found either. Since intraperitoneal injections every 8 h for an extended time period is rather burdensome for the animals and oral PTX is effective in patients with vascular diseases [39], we opted for oral administration in the current experiment. Because we did not find any beneficial effects of intraperitoneally administered PTX either, it seems unlikely that the absence of effects of PTX in the current study can be explained by this difference in experimental design.

PTX has also been used in clinical trials. Hoffman et al. [40] observed a shorter duration of required ventilator support, a shorter intensive care unit stay and a lower incidence of renal failure in patients at risk for developing MODS if they

were treated with intravenous PTX. Staubach et al. [41] found that continuous intravenous infusion of PTX resulted in an improved cardiopulmonary dysfunction in septic patients. However, serum TNF-α and IL-6 bioactivity were not different between the study groups, so the beneficial effects of PTX in this study could be the result of its rheological instead of its anti-inflammatory effects.

The results reported here do not support the hypothesis that PTX has beneficial effects on outcome in zymosan-induced murine MODS. One should be always be careful to extrapolate the results of animal studies to human pathology. However, the data at least constitute a warning regarding the potential (lack of) effictiveness of PTX in human patients at risk for MODS. Other agents that target multiple inflammatory mediators might yield better chances to provide treatment options and remain a topic of future investigation.

References

1. Seely AJ, Christou NV. Multiple organ dysfunction syndrome: exploring the paradigm of complex nonlinear systems. Crit Care Med 2000; 28:2193-2200.

2. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit Care Med 2001; 29:S99-106.

3. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

4. Goris RJ. MODS/SIRS: result of an overwhelming inflammatory response? World J Surg 1996; 20:418-421.

5. Jansen MJ, Hendriks T, Verhofstad AA, Lange W, Geeraedts-LM J, Goris RJ. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

6. Volman TJH, Goris RJ, van der Jagt M, van de Loo FAJ, Hendriks T. Organ damage in zymosan-induced multiple organ dysfunction syndrome in mice is not mediated by inducible nitric oxide synthase. Crit Care Med 2002; 30:1553-1559.

7. Demling R, Lalonde C, Youn YK, Daryani R, Campbell C, Knox J. Lung oxidant changes after zymosan peritonitis: relationship between physiologic and biochemical changes. Am Rev Respir Dis 1992; 146:1272-1278.

8. Shayevitz JR, Miller C, Johnson KJ, Rodriguez JL. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. Shock 1995; 4:389-396.

9. Cuzzocrea S, McDonald MC, Mazzon E, Filipe HM, Centorrino T, Lepore V, Terranova ML, Ciccolo A, Caputi AP, Thiemermann C. Beneficial effects of tempol, a membrane-permeable radical scavenger, on the multiple organ failure induced by zymosan in the rat. Crit Care Med 2001; 29:102-111.

10. Jansen MJ, Hendriks T, Vogels MT, Van der Meer JW, Goris RJ. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

11. Volman TJ, Goris RJ, Van der Meer JW, Hendriks T. Tissue- and time-dependent upregulation of cytokine mRNA in a murine model for the multiple organ dysfunction syndrome. Ann Surg 2004; 240:142-150.

12. Jansen MJ, Hendriks T, Knapen MF, van Kempen LC, Van der Meer JW, Goris RJ. Chlorpromazine down-regulates tumor necrosis factor-alpha and attenuates experimental multiple organ dysfunction syndrome in mice. Crit Care Med 1998; 26:1244-1250.

13. Jansen MJ, Hendriks T, Hermsen R, Van der Meer JW, Goris RJ. A monoclonal antibody against tumour necrosis factor-alpha improves survival in experimental multiple organ dysfunction syndrome. Cytokine 1998; 10:904-910.

14. Jansen MJ, Hendriks T, de Man BM, Van der Meer JW, Goris RJ. Interleukin 10 mitigates the development of the zymosan-induced multiple organ dysfunction syndrome in mice. Cytokine 1999; 11:713-721.

15. Dettelbach HR, Aviado DM. Clinical pharmacology of pentoxifylline with special reference to its hemorrheologic effect for the treatment of intermittent claudication. J Clin Pharmacol 1985; 25:8-26.

16. Ward A, Clissold SP. Pentoxifylline. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic efficacy. Drugs 1987; 34:50-97.

17. Lundblad R, Ekstrom P, Giercksky KE. Pentoxifylline improves survival and reduces tumor necrosis factor, interleukin-6, and endothelin-1 in fulminant intra-abdominal sepsis in rats. Shock 1995; 3:210-215.

18. Koo DJ, Yoo P, Cioffi WG, Bland KI, Chaudry IH, Wang P. Mechanism of the beneficial effects of pentoxifylline during sepsis: maintenance of adrenomedullin responsiveness and downregulation of proinflammatory cytokines. J Surg Res 2000; 91:70-76.

19. Tong Z, Chen B, Dai H, Bauer PC, Guzman J, Costabel U. Extrinsic allergic alveolitis: inhibitory effects of pentoxifylline on cytokine production by alveolar macrophages. Ann Allergy Asthma Immunol 2004; 92:234-239.

20. Doherty GM, Jensen JC, Alexander HR, Buresh CM, Norton JA. Pentoxifylline suppression of tumor necrosis factor gene transcription. Surgery 1991; 110:192-198.

21. Bahra PS, Rainger GE, Wautier JL, Nash GB. Effects of pentoxifylline on the different steps during adhesion and transendothelial migration of flowing neutrophils. Cell Biochem Funct 2001; 19:249-257.

22. Tighe D, Moss R, Hynd J, Boghossian S, al Saady N, Heath MF, Bennett ED. Pretreatment with pentoxifylline improves the hemodynamic and histologic changes and decreases neutrophil adhesiveness in a pig fecal peritonitis model. Crit Care Med 1990; 18:184-189.

23. Sullivan GW, Carper HT, Novick WJJ, Mandell GL. Inhibition of the inflammatory action of interleukin-1 and tumor necrosis factor (alpha) on neutrophil function by pentoxifylline. Infect Immun 1988; 56:1722-1729.

24. Beshay E, Croze F, Prud'homme GJ. The phosphodiesterase inhibitors pentoxifylline and rolipram suppress macrophage activation and nitric oxide production in vitro and in vivo. Clin Immunol 2001; 98:272-279.

25. Volman TJH, Hendriks T, Verhofstad AA, Kullberg BJ, Goris RJ. Improved survival of TNF-deficient mice during the zymosan-induced multiple organ dysfunction syndrome. Shock 2002; 17:468-472.

26. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.

27. Bone RC. Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. Crit Care Med 1996; 24:163-172.

28. Malazdrewich C, Thumbikat P, Abrahamsen MS, Maheswaran SK. Pharmacological inhibition of Mannheimia haemolytica lipopolysaccharide and leukotoxin-induced cytokine expression in bovine alveolar macrophages. Microb Pathog 2004; 36:159-169.

29. Poulakis N, Androutsos G, Kazi D, Bastas A, Provata A, Bitsakou C, Kontozoglou T, Polyzogopoulou C, Tassiopoulou A. The differential effect of pentoxifylline on cytokine production by alveolar macrophages and its clinical implications. Respir Med 1999; 93:52-57.

30. Myers MJ, Baarsch MJ, Murtaugh MP. Effects of pentoxifylline on inflammatory cytokine expression and acute pleuropneumonia in swine. Immunobiology 2002; 205:17-34.

31. Tong Z, Dai H, Chen B, Abdoh Z, Guzman J, Costabel U. Inhibition of cytokine release from alveolar macrophages in pulmonary sarcoidosis by pentoxifylline: comparison with dexamethasone. Chest 2003; 124:1526-1532.

32. Honess DJ, Dennis IF, Bleehen NM. Pentoxifylline: its pharmacokinetics and ability to improve tumour perfusion and radiosensitivity in mice. Radiother Oncol 1993; 28:208-218.

33. Miller K, Louie A, Baltch AL, Smith RP, Davis PJ, Gordon MA. Pharmacokinetics of pentoxifylline and its metabolites in healthy mice and in mice infected with Candida albicans. Antimicrob Agents Chemother 1998; 42:2405-2409.

34. DeVault GAJ, Kohan DE, Nelson EW, Holman JMJ. The effects of oral pentoxifylline on the cytokine release syndrome during inductive OKT3. Transplantation 1994; 57:532-540.

35. Kremsner PG, Grundmann H, Neifer S, Sliwa K, Sahlmuller G, Hegenscheid B, Bienzle U. Pentoxifylline prevents murine cerebral malaria. J Infect Dis 1991; 164:605-608.

36. Edwards MJ, Heniford BT, Klar EA, Doak KW, Miller FN. Pentoxifylline inhibits interleukin-2-induced toxicity in C57BL/6 mice but preserves antitumor efficacy. J Clin Invest 1992; 90:637-641.

37. Louie A, Baltch AL, Franke MA, Ritz WJ, Smith RP, Singh JK, Gordon MA. Effect of pentoxifylline on the course of systemic Candida albicans infection in mice. J Antimicrob Chemother 1996; 37:943-954.

38. Diaz Granados N, Howe K, Lu J, McKay DM. Dextran sulfate sodium-induced colonic histopathology, but not altered epithelial ion transport, is reduced by inhibition of phosphodiesterase activity. Am J Pathol 2000; 156:2169-2177.

39. Sha MC, Callahan CM. The efficacy of pentoxifylline in the treatment of vascular dementia: a systematic review. Alzheimer Dis Assoc Disord 2003; 17:46-54.

40. Hoffmann H, Markewitz A, Kreuzer E, Reichert K, Jochum M, Faist E. Pentoxifylline decreases the incidence of multiple organ failure in patients after major cardio-thoracic surgery. Shock 1998; 9:235-240.

41. Staubach KH, Schroder J, Stuber F, Gehrke K, Traumann E, Zabel P. Effect of pentoxifylline in severe sepsis: results of a randomized, double-blind, placebo-controlled study. Arch Surg 1998; 133:94-100.

6

Organ damage in zymosan-induced multiple organ dysfunction syndrome in mice is not mediated by inducible nitric oxide synthase.

Volman,TJH; Goris,RJA; Van der Jagt,M; Van de Loo,FAJ; Hendriks,T

Crit-Care-Med. 2002 Jul; 30(7): 1553-9

ABSTRACT

OBJECTIVE: To examine the role of inducible nitric oxide synthase (iNOS) in the development of the multiple organ dysfunction syndrome (MODS) in a murine model by using either a selective iNOS inhibitor or iNOS knockout mice.
DESIGN: Prospective randomized laboratory study.
SETTING: Central animal laboratory and experimental laboratory.
SUBJECTS: A total of 50 inbred C57BL/6 mice, 39 iNOS knockout (-/-) mice and 30 wildtype (+/+) mice, 7 to 9 weeks old, weighing 20-25 g.
INTERVENTIONS: Mice received an aseptic intraperitoneal injection of 40 μg lipopolysaccharide (LPS) followed by zymosan at a dose of 1 mg/g body weight 6 days later (day 0). In experiment I, C57BL/6 mice additionally received intraperitoneal injections with 5 mg aminoguanidine or saline every 12h, from 4 days after the injection of zymosan onward. In experiment II, both iNOS-/- mice and corresponding wildtype (iNOS+/+) mice were treated with LPS and zymosan.
MEASUREMENTS AND MAIN RESULTS:
In all animals the injection of zymosan induced an acute peritonitis, followed by an apparent recovery. From approximately day 6 onwards, animals entered the third - MODS-like - phase, indicated by weight loss, a decrease in body temperature and significant mortality.
Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and immunochemistry revealed a strongly increased expression of iNOS mRNA and iNOS protein in livers of mice in the last phase. However, neither the *in vivo* administration of aminoguanidine to C57BL/6 mice, nor the complete absence of iNOS enzyme (iNOS-/- mice) had a beneficial effect on survival rate, body temperature, or body weight. In addition, relative lung-, liver- and spleen weights and lung scores were not different between experimental groups.

CONCLUSIONS: The current results strongly argue against an essential and causative role of iNOS in the development of organ damage in our murine model of MODS.

INTRODUCTION

The development of the multiple organ dysfunction syndrome (MODS) by patients suffering from multiple trauma, shock, pancreatitis, or peritonitis is a major problem in the intensive care unit. MODS is a syndrome characterized by the progressive deterioration of the function of various organs, starting mostly with the adult respiratory distress syndrome (ARDS), followed by dysfunction c.q. failure of the liver, gut, kidneys, and the circulation. Once MODS develops, the morbidity and mortality remain high. Support of normal organ function is currently the best way to prevent MODS [1-3].

It is now generally accepted that MODS results from an uncontrolled, systemic inflammatory response rather than infection per se [4-9]. The development of adequate treatment protocols depends on further elucidation of the mechanisms and mediators involved. This type of research requires invasive procedures that cannot be easily performed on humans. Therefore we have developed an animal model for MODS [10,11], which has been adopted by several laboratories, sometimes in modified versions [12-14]. In this model, intraperitoneal administration of zymosan in a paraffin oil suspension leads to a three-phasic illness, consisting of an episode of acute illness, followed by a period of apparent recovery and, finally, the development of MODS-like symptoms.

Nitric oxide (NO) is a reactive and readily diffusible gas produced from L-arginine by one of three nitric oxide synthase (NOS) enzymes, namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). eNOS is normally present in vascular endothelium, nNOS is found in neurones of the brain and in the enteric nervous system. The inducible isoform of the enzyme, iNOS, is found in endothelium, epithelium, and inflammatory cells. iNOS is induced by cytokines and lipopolysaccharide (LPS), and produces large quantities of NO for extended periods of time. In the early 1990s the paradigm was put forward that the constitutively expressed eNOS and nNOS are critical to

normal physiology, whereas induction of iNOS causes tissue injury [15,16]. Although NO per se may not be detrimental, it can, at least *in vitro*, react with superoxide to produce peroxynitrite, which has been shown to induce direct cellular injury [16-18].

During inflammation and shock, proinflammatory cytokines and bacterial wall components may induce iNOS. It has been proposed that in these conditions the NO produced may damage tissue, which ultimately leads to organ failure [19-21]. Furthermore, iNOS-derived NO (or peroxynitrite) may also contribute to tissue damage indirectly by inducing proinflammatory mediators [18].

Since the development of MODS is thought to be driven by an uncontrolled inflammatory reaction, upregulation of iNOS may be expected. Indeed, in previous experiments with zymosan-induced MODS in mice we found that the capacity of peritoneal macrophages to produce NO is strongly increased [22]. This raises the question if iNOS is essential in the pathophysiology of MODS. Therefore, we examined the course of MODS – in our murine model - both in the presence of an iNOS inhibitor and in the complete absence of iNOS, by using iNOS knockout mice.

MATERIAL AND METHODS

Animals

Experiments were performed by using 50 inbred C57BL/6 mice (Charles River, WIGA, Germany), 39 iNOS knockout (-/-) mice and 30 wildtype (+/+) mice, 7 to 9 weeks old, weighing 20-25 g. INOS-/- mice were generated as described previously [23]. iNOS-/- and iNOS+/+ mice were intercross progeny (C57Bl/6x129Sv). Colonies were maintained at the Central Animal Laboratory (Nijmegen, Netherlands). All mice were housed under specific pathogen-free conditions during breeding and experiments. Animals were housed in filter-top

cages and fed a standard chow and acidified water at libitum. iNOS-/- deficiency of mice was routinely controlled during breeding by RT-PCR using primers bridging the neo cassette replacement of the first 4 exons of the iNOS gene: upstream 5'-CCC TAA GAG TCA CCA AAA TGG-3', downstream 5'-CTA CAG TTC CGA GCG TCA AA-3'. The animals were fed standard chow (Hope Farms RBM-H, Woerden, the Netherlands) and acidified water ad libitum. The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 5 days. The experiments were approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Nijmegen.

Zymosan-induced generalized inflammation

The animal model for MODS has been characterized previously [11]. Briefly, mice received an aseptic intraperitoneal injection of 40 μg lipopolysaccharide (LPS) (E.coli, Sigma, St Louis) dissolved in 200 μl PBS, six days prior to zymosan administration. Zymosan A (Sigma, St. Louis, MO) was sterilized by gamma radiation (5 kGray) and homogenously suspended in sterile paraffin oil (25 mg/ml) by high frequency vibration during 1 h. After sonification, the suspension was sterilized again in a waterbath at 100°C for 80 min. All suspensions were freshly made before use. Zymosan was given in a dose of 1 mg/g body weight.

Experimental design of in vivo studies

In experiment I, 37 C57BL/6 mice were used: 17 mice received intraperitoneal injections of 5 mg aminoguanidine in 100 μl 0.9% NaCl every 12 h, starting 4 days after the injection of zymosan, while another 17 mice received 100 μl of 0.9% NaCl only. Three mice did not receive zymosan or any other treatment and functioned as untreated controls. Survival, body weight and body temperature

were monitored. At day 14, surviving mice were killed by cervical dislocation and the lungs, liver, and spleen were collected and weighed. Lung damage was assessed macroscopically, using an arbitrary lung score: 0: no hemorrhages 1: hemorrhagic spots covering approximately 1-25% of the lung surface; 2: confluent hemorrhagic spots covering approximately 25-50% of the lung surface; 3: great areas of hemorrhaging visible covering 50-75% of the lung surface; 4: completely hemorrhagic lungs (75-100% of the surface covered with hemorrhages).

In experiment II, 24 iNOS-/- mice and 25 corresponding wildtype (iNOS+/+) mice were used. 3 iNOS-/- mice and 6 iNOS+/+ functioned as untreated controls. Survival, body weight and body temperature were monitored. At day 16, surviving mice were killed by cervical dislocation and the lungs, liver, and spleen were collected and weighed. Lung damage was assessed macroscopically.

In additional experiments with C57BL/6 mice, animals were killed at day 12 and lungs, liver, spleen and kidneys were collected. INOS gene expression was measured by quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and iNOS protein in liver tissue was detected by immunohistochemistry.

Immunohistochemistry

Tissues were fixed for 4 h in phosphate buffered 3.8% formaldehyde and 4 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, nonspecific adsorption was minimized by incubating the sections in 10% normal goat serum in phosphate-buffered saline for 15 min. The sections were then incubated with 1:50 dilution of primary rabbit anti-murine iNOS antibody (sc-650, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or with phosphate-buffered saline (control sections) for 1 h at room temperature. Specific labeling

was detected with a biotin-conjugated goat anti-rabbit immunoglobulin G (BA1000, Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin alkaline phosphatase complex. Tissues were counterstained with methyl green (Sigma, St. Louis, MO, USA).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

After collection, organs were snap-frozen in liquid nitrogen and stored at –80°C. Frozen organs were homogenized and total RNA was extracted with the guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacci [24]. RNA concentration was determined by spectrophotometric measurements with a GeneQuant II RNA/DNA calculator (Amersham Pharmacia Biotech Ltd). The purity of precipitated total RNA was ascertained by 1% agarose gel electrophoresis.

Isolated RNA was used as a template for the production of first strand cDNA using random hexamers. Relative quantification of iNOS mRNA was performed with a TaqMan real-time RT-polymerase chain reaction (PCR) assay on a ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc.). The standard curve was generated using RNA from a mouse macrophage cell line (J774-A1) that was stimulated with LPS (4 µg/ml) and human IFN-γ (0.85 µg/ml) for 8 h. iNOS gene expression was evaluated with the constitutively expressed 18S ribosomal RNA as endogenous control. Oligonucleotide primers and TaqMan probe were designed with use of the Primer Express 1.0 software (Perkin-Elmer Applied Biosystems Inc).

The PCR conditions were as follows: for amplification of the iNOS product, 25 ng of cDNA was added to the PCR mixture consisting of 25 µl TaqMan Mastermix (Perkin Elmer Corporation, Foster City, CA, USA), 12.5 pmol fluorescent probe and 15 pmol of each primer in a final volume of 50 µl. For amplification of the 18S rRNA product, the PCR mixture consisted of 25 µl

TaqMan Mastermix and 2.5 µl of pre-developed TaqMan 18S rRNA control kit (Perkin-Elmer). Again, the final volume was 50 µl. Thermal cycling conditions were 2 min at 50°C and 10 min of initial denaturation at 95°C to activate *Taq* Gold polymerase, followed by 40 cycles of 2-step PCR consisting of 15 s at 95°C and 1 min at 60°C. Samples and standards were measured in duplicate.

Macrophage nitrite production

The effects of aminoguanidine on LPS-induced nitrite production were examined both in a mouse macrophage cell line and in isolated mouse peritoneal macrophages.

Cells from the cell line J774.A1 were resuspended in Dubecco's Modified Eagle Medium (Nutrient mixture F-12, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum, L-glutamine (0.29 mg/ml), penicillin (100 U /ml) and streptomycin (100 µg/ml) to a concentration of $1*10^6$ cells/ml. The cell suspension was transferred to a 96 wells plate (250 µl/well).

Peritoneal macrophages were collected as follows: after cervical dislocation, 4 ml of ice-cold sterile phosphate buffered saline (PBS) containing 0.38% citrate was injected into the peritoneum of C57BL/6 mice. The PBS was withdrawn under aseptic conditions, cells were washed twice in cold PBS, and resuspended in RPMI1640 medium (Life Technologies, Paisley, UK) supplemented with Na-pyruvate (0.11 mg/ml), Gentamycinsulphate (0.05 mg/ml) and L-glutamine (0.29 mg/ml) to a final concentration of $1*10^6$ cells/ml.

After allowing the cells to attach for 24 h, the supernatants were removed and the cells were incubated for 24 h at 37°C (5% CO_2) with medium containing 10 µg LPS/ml with or without aminoguanidine (in a final concentration of 0.1 or 1.0 mM). Then 30 µl aliquots from these cultures were incubated for 20 minutes at room temperature with 0.005 U nitrate reductase and 7.5 µg NADPH in a

total volume of 50 µl. Subsequently these aliquots were incubated for 10 min at room temperature with 50 µl of Griess reagent. The absorbancy at 540 nm was determined in a microtiter plate reader. Nitrite was quantified using $NaNO_2$ as a standard. Standards were measured in triplicate, samples in quadruplicate.

Statistical analysis

Statistical significance of differences in mortality between experimental groups was analyzed using Fisher's exact test. Statistical significances of differences in body temperature and –weight and of differences in lung score were analyzed using the Mann-Whitney U test. One-way ANOVA was used to test whether variation among relative organ weights of experimental groups was greater than expected by chance. (According to the Kolmogorov-Smirnov-method, data were sampled from populations that follow Gaussian distributions.) When significant, Tukey-Kramer post-tests were performed to assess statistical significance between individual groups. P-values smaller than 0.05 were considered significant.

RESULTS

Zymosan-induced generalized inflammation

After the injection of LPS on day –6, all animals became ill for one day, indicated by a transient loss in body weight. By the time they received zymosan (day 0), the animals had recovered completely. The injection of zymosan induced an acute peritonitis rendering all animals very ill during the first two days, as reflected by a ruffled fur, diarrhea, lethargic behavior and a decrease in body weight and temperature. During the second phase of the illness (day 3 to 5) the condition of the surviving animals appeared to return to normal, reflected by

the display of natural curiosity, grooming, a smooth fur, and disappearance of diarrhea. Body temperature was restored and animals gained weight again. From approximately day 6 onwards, animals entered the third - MODS-like - phase, indicated by lethargic behavior, breathing difficulties, weight loss and a decrease in body temperature.

iNOS expression in liver tissue

Quantitative RT-PCR revealed a strongly increased expression of iNOS mRNA in livers of C57BL/6 mice in phase 3. Average normalized iNOS expression in livers of untreated control mice was 0.03±0.01, while in livers of mice that were in phase 3 this was 75.27±19.42 (Table 1). Also, in 2 out of 4 livers of mice that were killed in phase 3, expression of iNOS protein could be detected by immunohistochemistry, whereas this was not the case in livers of untreated controls or in livers of iNOS-/- mice that were killed in phase 3 (Figure 1). The strong upregulation of the iNOS gene in phase 3 seemed to be limited to liver tissue. In lungs, spleen, and kidneys, iNOS mRNA expression was somewhat upregulated in phase 3, but this upregulation was below the threshold of accurate quantification (data not shown).

Effect of aminoguanidine on zymosan-induced MODS

In both J774-A1 cells and isolated mouse peritoneal macrophages, the *in vitro* administration of aminoguanidine inhibited the LPS-induced nitrite production in a dose-dependent fashion. In both cell types, the highest dose of aminoguanidine effectively abolished the LPS-induced nitrite production since the resulting concentrations were similar to those produced in the absence of LPS (Figure 2).

Table 1. Expression of iNOS mRNA in liver from untreated C57BL/6 control mice and zymosan-treated C57BL/6 mice that were in the (MODS-like) phase 3 of the model.

Sample	**Cycle threshold value**	**Normalized iNOS expression[a]**
Control mouse 1	39.25	0.04
Control mouse 2	39.57	0.02
Control mouse 3	39.53	0.02
Phase 3 mouse 1	27.58	80.07
Phase 3 mouse 2	28.56	76.03
Phase 3 mouse 3	28.83	69.71

[a] Cycle threshold (Ct) values for both iNOS and 18S rRNA expression were transformed to ng of cDNA by use of calibration curves for iNOS and 18S rRNA expression in the J774.A1 cell line. Subsequently, normalized iNOS expression was calculated as 100x(iNOS/18S rRNA). Samples and standards were measured in duplicate.

The *in vivo* administration of aminoguanidine in C57BL/6 mice, starting 4 days after the injection of zymosan, did not result in an improved survival rate as compared to animals that were injected with saline only (Figure 3A), survival rates being 59% and 53%, respectively. Also, there was no difference in average body temperature (33.4±2.9°C vs 33.7±2.5°C) and average relative body weight (83.4±12.3% vs 85.9±15.9%) between the aminoguanidine- and saline treated groups at day 14 (Figure 3B and 3C). Relative lung, liver and spleen weights were elevated in both groups compared to organs of untreated control animals, indicating organ damage. However, there were no significant differences in either organ weights or macroscopic lung score (Figure 4) between the aminoguanidine- and saline treated groups.

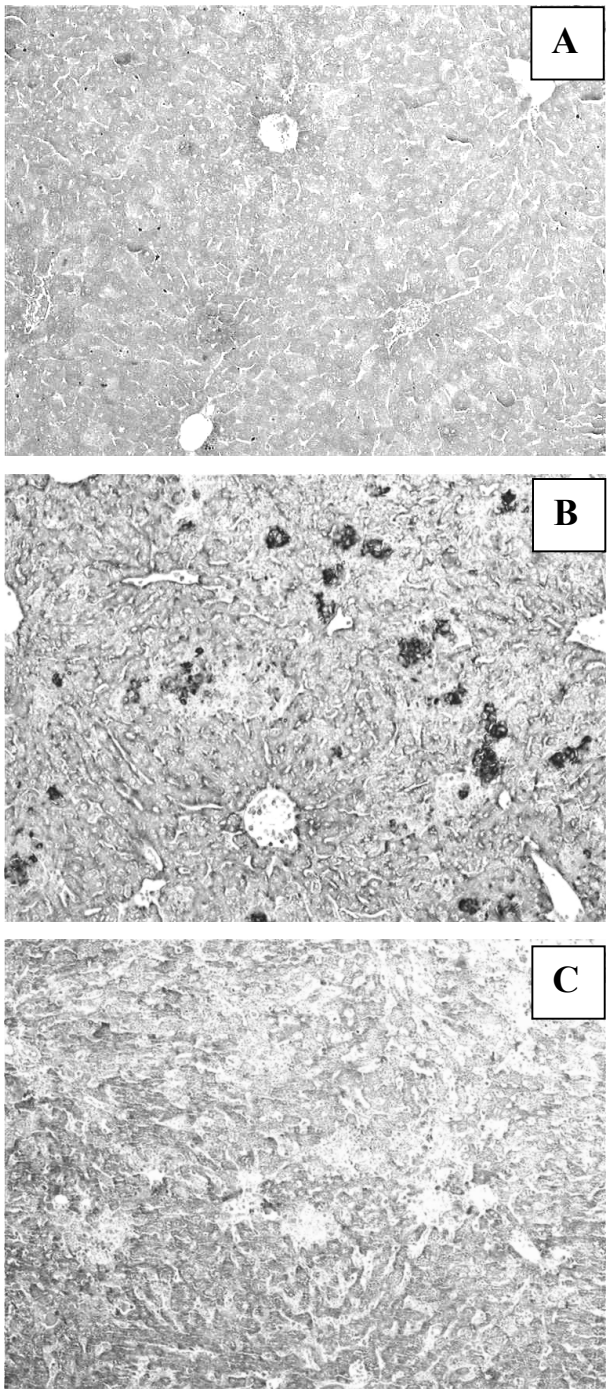

Figure 1. Immunohistochemical staining of iNOS protein in liver tissue. A: untreated C57BL/6 control mice; B: zymosan-treated C57BL/6 mice; C: zymosan-treated iNOS -/- mice. Livers of zymosan-treated mice were harvested in the third - MODS-like - phase of the model.

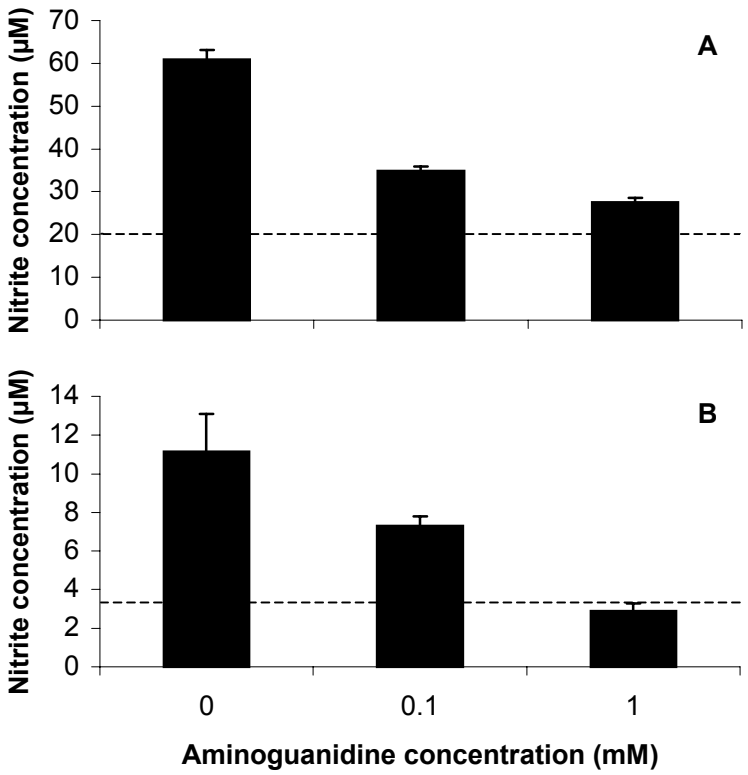

Figure 2. Nitrite production by J774.A1 cells (A) and isolated peritoneal macrophages (B). Cells were incubated for 24 h with LPS (10 µg/ml) and variable concentrations of aminoguanidine. Dotted lines represent basal nitrite concentration (produced in the absence of LPS). Values represent average + SD (n=4).

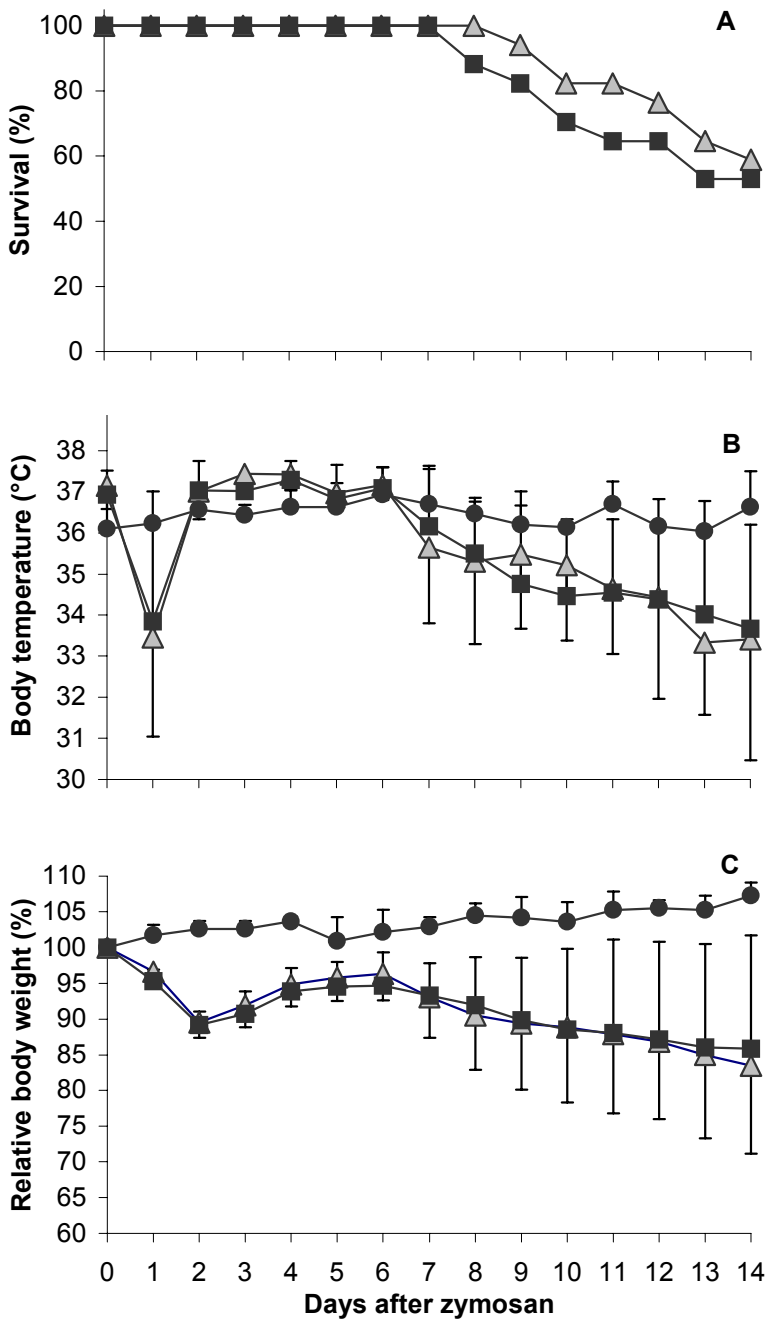

Figure 3. Effect of aminoguanidine administration on survival and clinical symptoms. Survival (A) of zymosan-treated C57BL/6 mice that received an ip injection of 5 mg aminoguanidine (▲, n=17) or saline (■, n=17) every 12 h, starting 4 days after the injection of zymosan. Average body temperature (B) and average relative body weight (C) of untreated control mice (●, n=3) and surviving zymosan-treated C57BL/6 mice that were treated with aminoguanidine (▲, n=10) or saline (■, n=9). Relative body weight was calculated as (body weight / body weight at day 0)x100%. Data represent average values + SD.

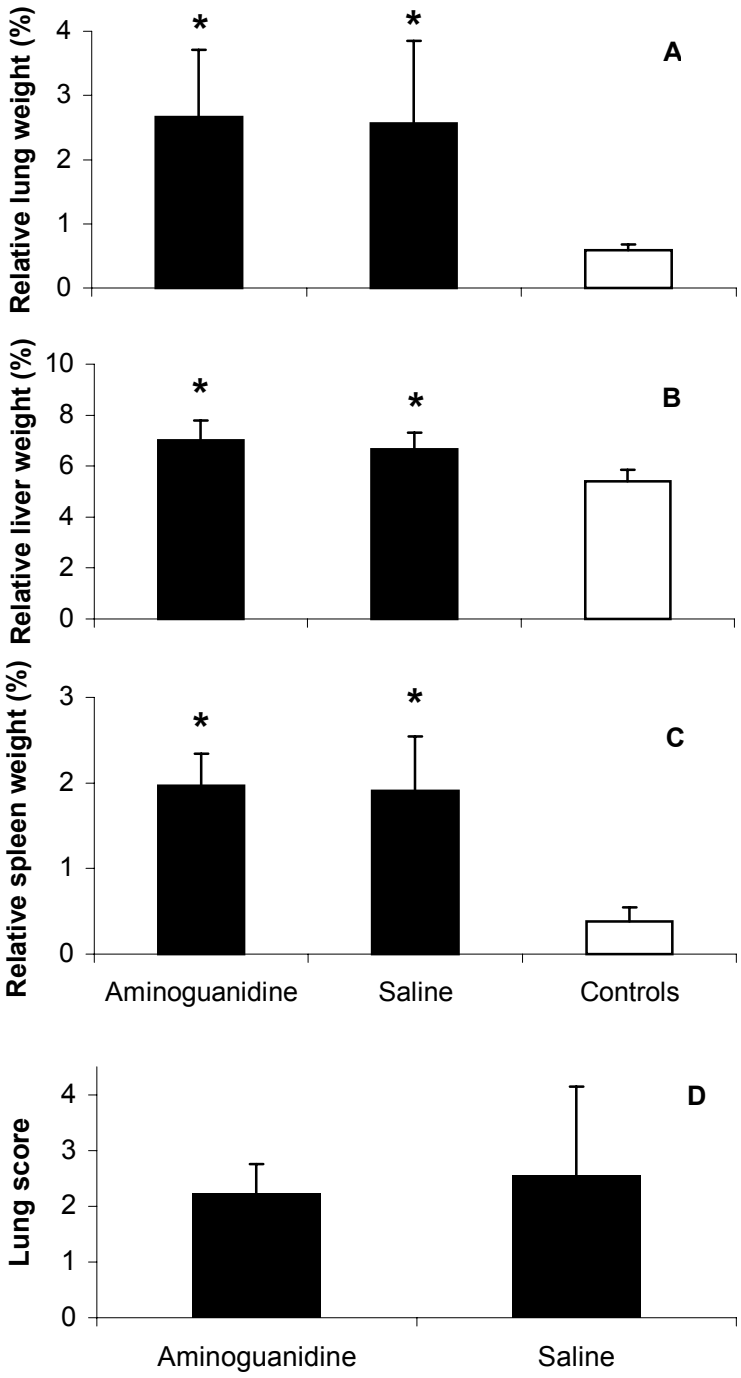

*Figure 4. Effect of aminoguanidine administration on organ weight and lung score. Average values (+SD) for relative lung (A), liver (B) and spleen (C) weight and lung score (D) in surviving C57BL/6 mice treated with aminoguanidine (n=10) or saline (n=9) and in untreated control mice (n=3). Relative organ weight was calculated as (organ weight / body weight)x100%. Lung scores in control mice were 0. * $p<0.05$ versus controls.*

Development of MODS in iNOS-/- mice

INOS knockout (-/-) mice and their corresponding wildtypes (+/+) also exhibited a similar course of illness. Survival rates of iNOS-/- and iNOS+/+ mice at day 16 were equal at 29% (Figure 5A). Also, the course of body temperature and body weight was similar in both groups. Although surviving iNOS-/- mice displayed a lower average body temperature (28.1±4.3°C vs 31.6±2.3°C, figure 5B) and a lower average relative body weight (73.9±6.2% vs 82.0±6.8%, figure 5C) at day 16 than the iNOS+/+ mice, these differences remained non-significant. Relative organ weights were elevated in both groups, as compared to organs of untreated control animals, although the difference in relative liver weight between iNOS-/- and untreated control mice did not reach statistical significance. There were, however, no significant differences in organ weights and macroscopic lung scores between iNOS-/- and iNOS+/+ mice (Figure 6).

DISCUSSION

The current results strongly argue against an essential and causative role of iNOS in the development of organ damage in our murine model of MODS. Neither treatment *in vivo* with an inhibitor of iNOS activity nor the complete absence of iNOS in the knockout mice leads to improved survival, abation of clinical signs for illness or attenuation of organ damage.
In our model, intraperitoneal administration of zymosan to rodents leads to an immediate shock-like state, followed by a short period of apparent recovery. Thereafter, the clinical condition of the animals deterioriates again with time, concomitant to a gradual development of organ dysfunction. Organ damage is characterized by histopathological changes and abnormal values of serum markers [11]. In addition, there are signs of generalized inflammation [22,25]. As such, the animals display a number of characteristics commonly observed during

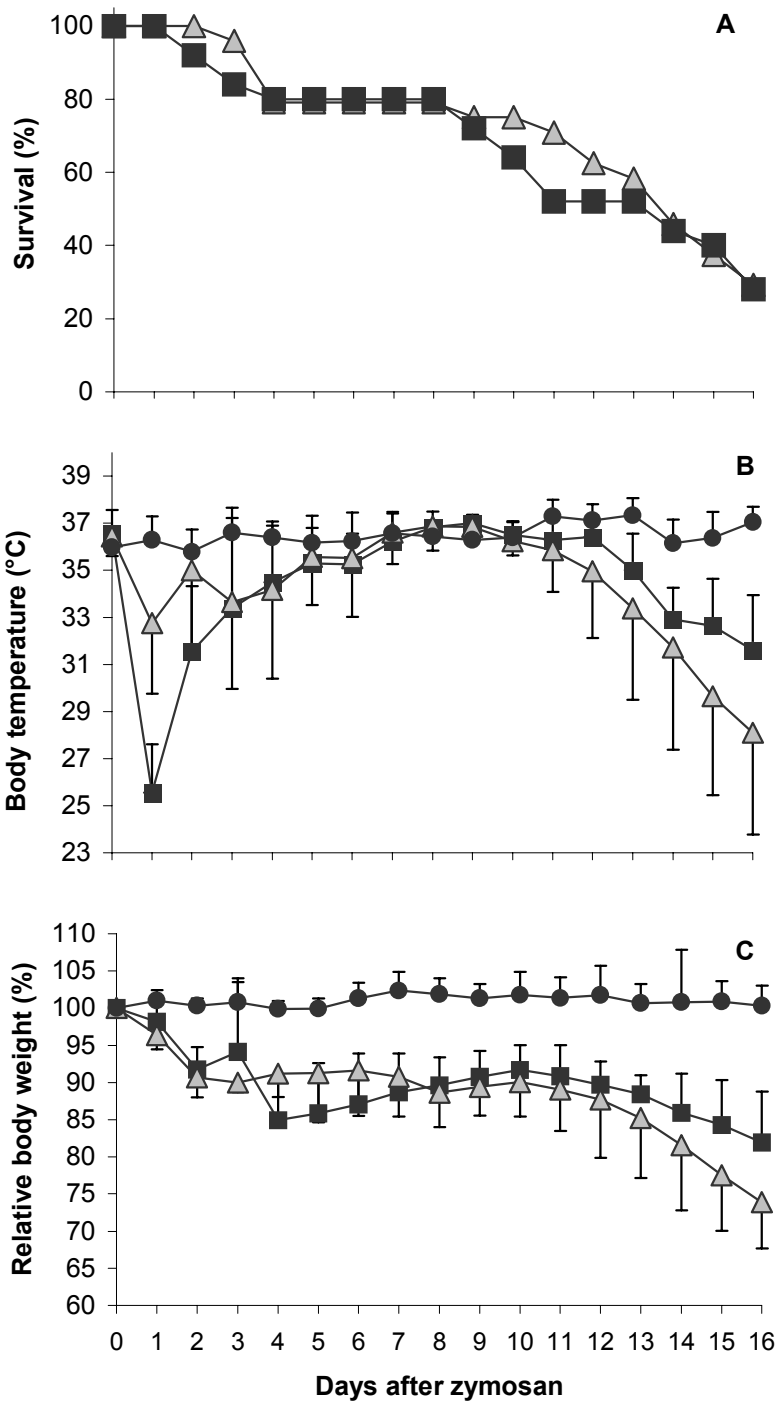

Figure 5. Survival and clinical symptoms in iNOS knockout and wildtype mice. Survival (A) in zymosan-treated iNOS-/- (▲, n=24) and iNOS +/+ (■, n=25) mice. Average body temperature (B) and average relative body weight (C) of surviving zymosan-treated iNOS-/- (▲, n=7), iNOS+/+ (■, n=7) and untreated control mice (●, n=10). Relative body weight was calculated as (body weight / body weight at day 0)x100%. Data represent average values + SD.

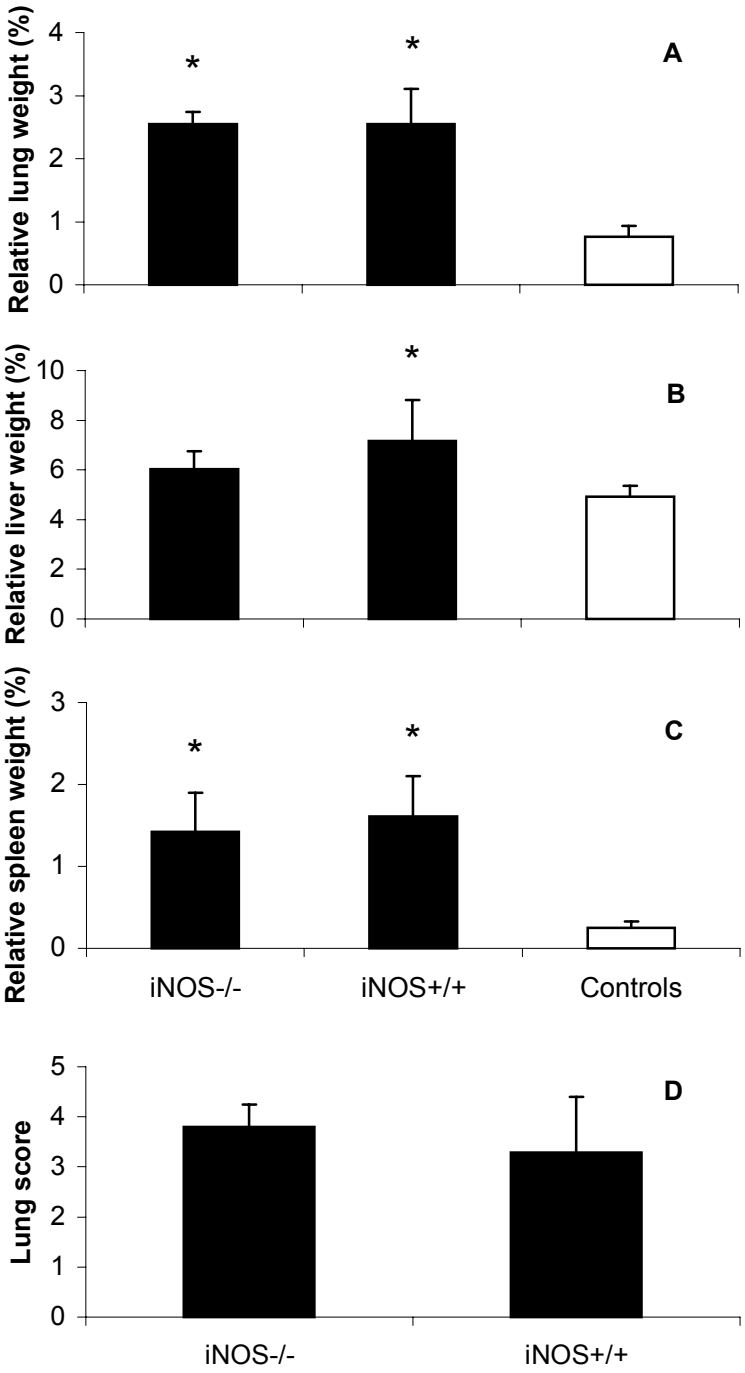

*Figure 6. Organ weight and lung score in iNOS knockout and wildtype mice. Average values (+SD) for relative lung (A), liver (B) and spleen (C) weight and lung score (D) in surviving iNOS-/- (n=7), iNOS+/+ (n=7), and untreated control mice (n=10) mice. Relative organ weight was calculated as (organ weight / body weight)x100%. Lung scores in control mice were 0. * $p<0.05$ versus controls.*

MODS. Because the early mortality in the ZIGI model recently increased dramatically in our experiments, most likely as the result of changing conditions within our central animal facility, we introduced a small modification to the ZIGI model. This modification consists of an aseptic intraperitoneal injection of 40 μg LPS six days before the intraperitoneal injection of zymosan. This way, the immune system of the animals is triggered before they receive zymosan, which prevents an excessive mortality rate in the first days after zymosan; the subsequent course of the model remains essentially unchanged. The effect of this LPS dose is limited and transient and does, in itself, not lead to any organ damage.

There exists ample evidence that iNOS plays an important role in the pathophysiology of several inflammatory disease states, such as chronic inflammatory bowel disorders [26], arthritis [27], and glomerulonephritis [28]. In our animal model, MODS is caused by zymosan-induced generalized inflammation. The present immunohistochemisty and quantitative RT-PCR data clearly indicate that in the third, MODS-like phase, both iNOS protein and mRNA are strongly upregulated in liver tissue. In addition, earlier experiments in our laboratory have shown that both unstimulated and LPS-stimulated NO production by peritoneal cells is elevated during this period [22]. These findings can be taken to suggest that NO produced by iNOS may play a role in the onset and propagation of MODS.

Aminoguanidine is a selective inhibitor of iNOS [29,30], although its affinity is not very high [31]. Results from our *in vitro* experiments show that aminoguanidine suppresses, dose-dependently, NO production in both a mouse macrophage cell line (as was reported previously [29]) and in isolated peritoneal macrophages. In the *in vivo* experiment, aminoguanidine has been given twice daily from day 4 after zymosan administration onwards when the first, shock-like phase, is over. The experiment was designed this way since intraperitoneal injections every 12 h with either aminoguanidine or saline starting immediately after the zymosan

dose cause a high mortality in the first days (data not shown). Apparently, during this phase the animals are too weak to cope with the stress resulting from the necessary manipulation twice every 24 h. Since MODS-like symptoms do not occur until day 6 or later, it seems unlikely that iNOS inhibition before day 4 will be of any influence on the clinical symptoms in the third phase. In the present study, 5 mg of aminoguanidine has been administered *in vivo* every 12h to C57BL/6 mice. This dose is comparable to doses used in earlier studies in other rodent models where iNOS inhibition led to an effective reduction of disease. For instance, Cross et al. [32] found significantly less pathology in mice treated with aminoguanidine in a model for multiple sclerosis, while Connor et al. [31] found that aminoguanidine is able to suppress joint inflammation associated with adjuvant-induced arthritis in rats in a dose-dependant manner. Altogether, the fact that aminoguanidine does not improve the course of MODS in our model constitutes the first argument against an essential role for iNOS in the development of this syndrome.

The second argument stems from the outcome of the experiment with iNOS-/- mice. These animals have been used before in a number of studies with models for diseases, where iNOS is involved in the pathology, and have often exhibited a reduced morbidity and mortality if compared to the wildtype iNOS+/+ mice. For instance, Ling et al. [33] found that kidneys of iNOS-/- mice were protected against acute ischemic renal failure, while Hollenberg et al. [34] observed a lower mortality in iNOS-/- mice than in iNOS+/+ mice in a septic shock model. Although there are also reports that iNOS-/- mice may display a higher morbidy and/or mortality in sepsis [35], the fact that there are differences in clinical parameters between iNOS-/- and iNOS+/+ mice indicates that iNOS-/- mice can be a useful tool for studying disease processes. However, in our model of zymosan-induced generalized inflammation, iNOS-/- mice do not show either improved survival or clinical condition, or a lesser degree of organ damage. This is in agreement with the results of Van de Loo et al. [36], who did not find a lesser

degree of zymosan-induced joint inflammation in iNOS-/- mice. In itself, this result does not unequivocally prove that the iNOS enzyme is not important in our model. It is possible that iNOS-/- mice develop mechanisms to compensate for their inability to produce iNOS. However, if taken together with the result from the aminoguanidine experiment, the current results make a strong argument against an essential role for iNOS in zymosan-induced MODS.

In seeming contrast to the present data, Cuzzocrea et al. [21,37] have reported experiments which suggest that NO is indeed involved in zymosan-induced organ damage. Administration of L-NAME, a nonselective NOS inhibitor, led to a reduced zymosan-induced organ injury and mortality in rats. This apparent discrepancy might be explained by the fact that their experiments were of much shorter duration than ours (48 h versus 12-17 days). Therefore, the model they used must be considered as a model for nonseptic shock rather than for MODS occurring as the result of a chronic systemic inflammatory response. In addition, since L-NAME is a nonselective inhibitor of NOS it cannot be ruled out that the beneficial effects of L-NAME administration are somehow related to the inhibition of eNOS/nNOS instead of iNOS.

Generally speaking, the paradigm that NO produced by iNOS causes tissue injury and therefore specific inhibition of this enzyme in inflammation is beneficial, may be too simplistic. In fact, NO can function as an antioxidant during the inflammatory response. Infiltrating neutrophils are an important source of oxidants. Under pathological conditions, the synthesis of reactive oxidant species by neutrophils is enhanced, resulting in lipid peroxidation and subsequent tissue damage, thus possibly contributing to the development of MODS. NO is also known to be an antiadhesive molecule, and by inhibiting the recruitment of neutrophils it might reduce oxidative stress. Furthermore, it reacts with superoxide to inactivate its biologic activity, and NO donors have been shown to reduce H_2O_2-induced injury and inflammation [15,16]. Still, in our model

the putative suppression of NO production by either iNOS inhibition or iNOS absence, does not lead to deterioration of the disease parameters either.
In previous experiments we have found that during the development of MODS the ability of peritoneal macrophages to produce both superoxide anion (O_2^-) and NO is elevated [22]. Apart from being an oxidant species itself, O_2^- can induce the formation of the hydroxyl radical ($^{\cdot}OH$). Like peroxynitrite, hydroxyl radical is a key trigger of DNA single strand breakage, with the subsequent activation of poly(ADP-ribose) synthetase (PARS) resulting in cell damage [38]. Thus, the formation of O_2^- might be considered as a contributing factor in the development of MODS. However, administration of superoxide dismutase and catalase in the acute phase after zymosan administration did not improve subsequent organ damage observed in rats [39].
In conclusion, the results reported here do not support an important and causative role for iNOS in zymosan-induced organ damage in mice. Elucidation of the mechanisms directly responsible for the observed organ damage remains a topic for future investigation.

ACKNOWLEDGMENTS

We thank Ben de Man, Roger Lomme and Louis van der Locht for expert technical assistance.

References

1. Baue AE. MOF/MODS, SIRS: an update. Shock 1996; 6 Suppl 1:S1-5.

2. Baue AE. Multiple organ failure, multiple organ dysfunction syndrome, and the systemic inflammatory response syndrome-where do we stand? [editorial]. Shock 1994; 2:385-397.

3. Baue AE, Durham R, Faist E. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? Shock 1998; 10:79-89.

4. Lemaire LC, van Lanschot JJ, Stoutenbeek CP, van Deventer SJ, Wells CL, Gouma DJ. Bacterial translocation in multiple organ failure: cause or epiphenomenon still unproven. Br J Surg 1997; 84:1340-1350.

5. Davies MG, Hagen PO. Systemic inflammatory response syndrome. Br J Surg 1997; 84:920-935.

6. Bone RC. Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. Crit Care Med 1996; 24:163-172.

7. Beal AL, Cerra FB. Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction. JAMA 1994; 271:226-233.

8. Goris RJ, te-Boekhorst TP, Nuytinck JK, Gimbrere JS. Multiple-organ failure. Generalized autodestructive inflammation? Arch Surg 1985; 120:1109-1115.

9. Yao YM, Redl H, Bahrami S, Schlag G. The inflammatory basis of trauma/shock-associated multiple organ failure. Inflamm Res 1998; 47:201-210.

10. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

11. Jansen MJ, Hendriks T, Verhofstad AA, Lange W, Geeraedts-LM J, Goris RJ. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

12. Burdon D, Tiedje T, Pfeffer K, Vollmer E, Zabel P. The role of tumor necrosis factor in the development of multiple organ failure in a murine model. Crit Care Med 2000; 28:1962-1967.

13. Ferrer TJ, Webb JW, Wallace BH, Bridges CD, Palmer HE, Robertson RD, Cone JB. Interleukin-10 reduces morbidity and mortality in murine multiple organ dysfunction syndrome (MODS). J Surg Res 1998; 77:157-164.

14. Steinberg S, Flynn W, Kelley K, Bitzer L, Sharma P, Gutierrez C, Baxter J, Lalka D, Sands A, van Liew J, et a. Development of a bacteria-independent model of the multiple organ failure syndrome. Arch Surg 1989; 124:1390-1395.

15. Kubes P. Inducible nitric oxide synthase: a little bit of good in all of us. Gut 2000; 47:6-9.

16. Kubes P, McCafferty DM. Nitric oxide and intestinal inflammation. Am J Med 2000; 109:150-158.

17. Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis--an overview. Acta Anaesthesiol Scand 1999; 43:275-288.

18. Szabo C, Billiar TR. Novel roles of nitric oxide in hemorrhagic shock. Shock 1999; 12:1-9.

19. Szabo C, Southan GJ, Thiemermann C. Beneficial effects and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. Proc Natl Acad Sci U S A 1994; 91:12472-12476.

20. Thiemermann C, Ruetten H, Wu CC, Vane JR. The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. Br J Pharmacol 1995; 116:2845-2851.

21. Cuzzocrea S, Zingarelli B, Sautebin L, Rizzo A, Crisafulli C, Campo GM, Costantino G, Calapai G, Nava F, Di Rosa M, Caputi AP. Multiple organ failure following zymosan-induced peritonitis is mediated by nitric oxide. Shock 1997; 8:268-275.

22. Jansen MJ, Hendriks T, Huyben CM, Tax WJ, Van der Meer JW, Goris RJ. Increasing cytotoxic activity and production of reactive oxygen and nitrogen intermediates by peritoneal macrophages during the development of multiple organ dysfunction syndrome in mice. Scand J Immunol 1996; 44:361-368.

23. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, et a. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 1995; 81:641-650.

24. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.

25. Jansen MJ, Hendriks T, Vogels MT, Van der Meer JW, Goris RJ. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

26. Perner A, Rask MJ. Review article: the potential role of nitric oxide in chronic inflammatory bowel disorders. Aliment Pharmacol Ther 1999; 13:135-144.

27. Amin AR, Attur M, Abramson SB. Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. Curr Opin Rheumatol 1999; 11:202-209.

28. Ketteler M, Distler A. The role of nitric oxide in experimental glomerulonephritis. Kidney Blood Press Res 1996; 19:177-181.

29. Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR, Currie MG. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur J Pharmacol 1993; 233:119-125.

30. Griffiths MJ, Messent M, MacAllister RJ, Evans TW. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br J Pharmacol 1993; 110:963-968.

31. Connor JR, Manning PT, Settle SL, Moore WM, Jerome GM, Webber RK, Tjoeng FS, Currie MG. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur J Pharmacol 1995; 273:15-24.

32. Cross AH, Misko TP, Lin RF, Hickey WF, Trotter JL, Tilton RG. Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. J Clin Invest 1994; 93:2684-2690.

33. Ling H, Edelstein C, Gengaro P, Meng X, Lucia S, Knotek M, Wangsiripaisan A, Shi Y, Schrier R. Attenuation of renal ischemia-reperfusion injury in inducible nitric oxide synthase knockout mice. Am J Physiol 1999; 277:F383-F390.

34. Hollenberg SM, Broussard M, Osman J, Parrillo JE. Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase. Circ Res 2000; 86:774-778.

35. Cobb JP, Hotchkiss RS, Swanson PE, Chang K, Qiu Y, Laubach VE, Karl IE, Buchman TG. Inducible nitric oxide synthase (iNOS) gene deficiency increases the mortality of sepsis in mice. Surgery 1999; 126:438-442.

36. van de Loo FA, Arntz OJ, van Enckevort FH, van Lent PL, van den Berg WB. Reduced cartilage proteoglycan loss during zymosan-induced gonarthritis in NOS2-deficient mice and in anti-interleukin-1-treated wild-type mice with unabated joint inflammation. Arthritis Rheum 1998; 41:634-646.

37. Cuzzocrea S, Filippelli A, Zingarelli B, Falciani M, Caputi AP, Rossi F. Role of nitric oxide in a nonseptic shock model induced by zymosan in the rat. Shock 1997; 7:351-357.

38. Szabo C. Role of poly(ADP-ribose)synthetase in inflammation. Eur J Pharmacol 1998; 350:1-19.

39. van Bebber IP, Lieners CF, Koldewijn EL, Redl H, Goris RJ. Superoxide dismutase and catalase in an experimental model of multiple organ failure. J Surg Res 1992; 52:265-270.

7

Increased expression of matrix metalloproteinases in the murine zymosan-induced multiple organ dysfunction syndrome.

Volman,TJH; Goris,RJA; Lomme,RM; De Groot,J; Verhofstad,AAJ; Hendriks,T

J-Pathol. 2004 Aug; 203(4): 968-75

ABSTRACT

Matrix metalloproteinases (MMPs) have been implicated as mediators of tissue damage in several inflammatory diseases. Since the Multiple organ dysfunction syndrome (MODS) is thought to result from systemic inflammation, overactivation of MMPs could contribute to the organ damage observed. The expression and activity of several MMPs were studied in a murine model for MODS. 60 mice were given an aseptic intraperitoneal injection of lipopolysaccharide, followed after six days by zymosan. At days 2, 5, 8, 12 and 16 after the injection of zymosan, groups of mice were killed and the liver, lungs, spleen and kidneys were collected for either RNA extraction, gelatinase zymography and collagenase (MMP-1 and –13) assays (6 mice per time point) or immunohistochemistry (3 mice per time point). A group of 9 mice did not receive zymosan and acted as control group. The expression of MMP-2 mRNA in zymosan-treated mice was strongly upregulated in liver tissue only. For MMP-9, this was the case in all organs examined. Quantitative gelatin zymography demonstrated the near complete absence of any gelatinase activity in tissues from control mice. However, in the liver, lungs and especially the spleen of zymosan-treated animals, a significantly increased activity of proform and active MMP-2 and –9 was observed with time. Overall, MMP-1 and –13 activities were very low in all samples from liver and lung. In the spleen, however, high levels of MMP-1 and –13 were observed in zymosan-treated animals. Immunohistochemical staining for MMP-2 was detected in the liver and the spleen but not in lung and kidney tissue of zymosan-treated animals. Staining for MMP-9 could be detected in liver, lung and spleen tissues of zymosan-treated mice. For both MMPs, staining appeared to be limited to phagocytes. In conclusion, the data suggests a role for MMPs, especially MMP-9, in the pathogenesis of MODS.

INTRODUCTION

A major cause of death in the surgical intensive care unit is the multiple organ dysfunction syndrome (MODS). The cumulative sequence of progressive deterioration of organ systems starts mostly in the lungs and is followed by dysfunction of the liver, gut and kidneys, which organs are not necessarily involved in the primary disease [1,2]. This destructive pathway may be encountered after a variety of serious insults like major trauma or severe bacterial infection. Most likely, it is the result of an overwhelming host response resulting in systemic inflammation [1,3,4]. Once MODS develops, morbidity and mortality rates are high. Treatment for patients with MODS is still largely supportive. Further development of treatment and prevention modalities requires elucidation of the mechanisms and mediators involved. Such research requires invasive procedures and collection of tissue samples which are virtually impossible to perform on patients. Therefore, we have developed an animal model for MODS [3,5,6], which is used widely to study systemic inflammation in relation to organ failure [7-9]. Mice that receive intraperitoneal zymosan display a gradual development of MODS-like symptoms.

Disruption of tissue architecture will result in organ dysfunction. The extracellular matrix is central to tissue architecture and homeostasis. Remodelling of the extracellular matrix is largely mediated by enzymes from the matrix metalloproteinase (MMP) family, which consists of a growing number of secreted and membrane-bound metalloendopeptidases. During inflammation, they play an important role in the control of cellular interactions with their environment [10]. MMPs have been implicated as mediators of tissue damage in several inflammatory diseases, such as rheumatoid arthritis [11], osteoarthritis [12] and atherosclerosis [13]. MMPs have also been related to systemic inflammation. Several studies have suggested an important role for MMPs in the development of the adult respiratory distress syndrome (ARDS) [14,15] and it has been shown

recently that circulating MMP-9 concentrations are elevated in critically ill patients [16].

It seems entirely possible that overactivation of MMPs could contribute to the organ damage observed in MODS. Gene expression of MMPs is regulated by several factors, among them cytokines. Previous research in our laboratory has demonstrated that the development of MODS in mice is, at least in part, the result of overexpression of TNF-α [17,18] and other cytokines (Volman et al., unpublished data). Since proinflammatory cytokines are known to induce MMP production and secretion [19,20], MMP activity may be upregulated in the various tissues affected. If so, specific inhibition of these enzymes may be a new target for preventing organ dysfunction. The present study presents the first data on the expression and activity of several gelatinases and collagenases in the murine model for MODS.

METHODS

Zymosan-induced generalized inflammation

The zymosan-induced generalized inflammation model for MODS has been described extensively [3,5,6,17,18]. Zymosan was given intraperitoneally at a dose of 0.8 mg/g body weight.

The current experiment was performed using 69 C57BL/6 mice, 7 to 9 weeks old and weighing 20-25 g. The experiment was approved by the Animal Ethics Review Committee of Nijmegen University.

Experimental design

Mice were divided into three groups. Group 1 consisted of 50 animals: at days 2, 5, 8, 12 and 16 after the injection of zymosan, 6 mice were taken randomly,

killed by cervical dislocation and the liver, lungs, spleen and kidneys were collected for RNA extraction, gelatinase zymography and collagenase assays. Organs were snap-frozen in liquid nitrogen and stored at –80°C. At days 2, 5, 8, and 16 after zymosan, another 3 mice were killed and their organs were collected for immunohistochemistry. Group 2 consisted of 9 mice that did not receive zymosan and acted as controls. The organs of 6 control mice were collected for biochemistry, while those of the other 3 were used for immunohistochemistry. In all mice, the lung damage was assessed macroscopically, using an arbitrary lung score (see legend to Figure 2). To monitor the clinical course and mortality due to zymosan treatment, a group of 10 additional mice (group 3) was treated with LPS and zymosan and their body temperature, body weight and survival were monitored until day 16, when surviving animals were killed.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA extraction and cDNA synthesis were described previously [6]. Relative quantification of MMP-2 and MMP-9 mRNA was performed with a TaqMan real-time RT-polymerase chain reaction (PCR) assay on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Quantification of the amount of target in unknown samples was accomplished using a standard curve to determine the starting concentration of target. The standard curve for MMP-2 was generated using RNA from a mouse fibroblast cell line (NIH-3T3) after stimulation with human interleukin-1β (100 ng/ml) for 24 h. The standard curve for MMP-9 was generated using RNA from a mouse macrophage cell line (J774.A1) treated with LPS (1 ng/ml) for 12h. In order to correct for variations in cDNA concentrations between samples, MMP-2 and -9 gene expression was normalized by dividing the starting concentration of target by the starting concentration of constitutively expressed 18S ribosomal RNA (endogenous

control). The PCR conditions for target and 18S rRNA products were as described previously [6]. Since we were primarily interested in temporal changes in gene expression, results are expressed in a relative way. Upregulation of gene expression at any time point was calculated as the median of normalized gene expressions divided by the median of normalized gene expression in untreated control mice.

Oligonucleotide primers and TaqMan probe were designed with use of the Primer Express 1.0 software (Applied Biosystems). The sequence (5'-3') of forward primer, reverse primer, and probe were CTGGAATGCCATCCCTGATAA, CAAACTTCACGCTCTTGAGACTTT and TGAAGAAGTAGCTATGACCACCGCCCTG, respectively, for MMP-2. The respective sequences for MMP-9 were AAGGGGCGTGTCTGGAGATT, GGTACTGGAAGATGTCGTGTGAGT, and CCCAGAGCGTCATTCGCGTGGATA.

Zymography

Frozen organs were pulverized, extracted and dialysed as described previously [21]. Gelatin zymography was performed to quantify the presence of both activated and latent forms of the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in the tissue extracts, as described previously [21].

Collagenase assays

A selection of tissue extracts (liver, lungs, and spleen extracts of control mice and mice killed at days 8 and 12 after zymosan, n=5 per group) were analysed for the collagenases MMP-1 and MMP-13. MMP activity was measured using quenched fluorogenic peptide substrates that were synthesized according to the method described by Drijfhout *et al*. [22]. Fluorescein was coupled to the peptides

via the cystein's thiol function using iodacetamide-fluorescein. All MMP assays were performed in Tris buffer (50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 1 μM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3; pH 7.5) in the presence of EDTA-free general proteinase inhibitor cocktail Complete (Roche, Mannheim, Germany) to prevent conversion of the fluorogenic substrates by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence or absence of the synthetic MMP inhibitor BB94. MMP-1 activity was determined [23] with the selective fluorogenic substrate TNO113-F (5 μM). MMP-13 activity was determined [24] using the selective fluorogenic substrate TNO013-F (5 μM). The rate of substrate conversion (RFU /s; relative fluorescence units per second) at 30°C (λ_{ex} 485 nm, λ_{em} 530 nm; Cytofluor4000; PerSeptive Biosystems) was normalized to the amount of protein present in the tissue extract and expressed as RFU/s per mg protein.

Immunohistochemistry

Tissues were fixed in phosphate buffered 3.8% formaldehyde for 4 h at room temperature. From paraffin-embedded tissues 4 μm sections were prepared. After deparaffinization, endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate-buffered saline. Nonspecific adsorption was minimized by preincubating the sections in 20% normal horse serum in phosphate-buffered saline for 10 min. The sections were then incubated overnight at 4^0C with 1:200 dilution of primary goat anti-human MMP-2, 1:400 goat anti-mouse MMP-9 antibody (sc-6838 and sc-6841, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or phosphate-buffered saline (control sections). Labeling was detected with a biotin-conjugated horse anti-goat immunoglobulin G serum (BA9500, Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin peroxidase

complex. Tissues were counterstained with hematoxylin (Klinipath, Duiven, the Netherlands). All sections for MMP-2 and MMP-9 were stained simultaneously.

RESULTS

Clinical course

The injection of zymosan induced an acute peritonitis rendering all animals very ill during the first two days, as reflected by a ruffled fur, diarrhea, lethargic behavior and a decrease in body weight and temperature (Figure 1). During the second phase of the illness (day 3 to 5) the condition of the surviving animals appeared to return to normal, reflected by the display of natural curiosity, grooming, a smooth fur, and disappearance of diarrhea. Body temperature was restored and animals gained weight again. From approximately day 6 onwards, animals entered the third - MODS-like - phase, indicated by lethargic behavior, breathing difficulties, weight loss and a decrease in body temperature. The progression of lung damage, which is reflected by an increased lung score from day 5 onwards, is illustrated in figure 2.

mRNA expression

In the liver, mRNA for both MMP-2 and -9 was strongly upregulated compared to untreated control animals. This was especially the case in the third, MODS-like, phase of the model, where the expression of both MMP-2 and -9 mRNA was 15 times higher than in control animals (Figure 3A). Figure 3 gives, for reasons of clarity, median values only. In order to demonstrate the degree of variation observed between animals, the expression of MMP-2 and -9 mRNA in the liver of all individual animals is illustrated in Figure 4. In the lungs there was no upregulation of MMP-2 mRNA, but MMP-9 mRNA levels were 15 times

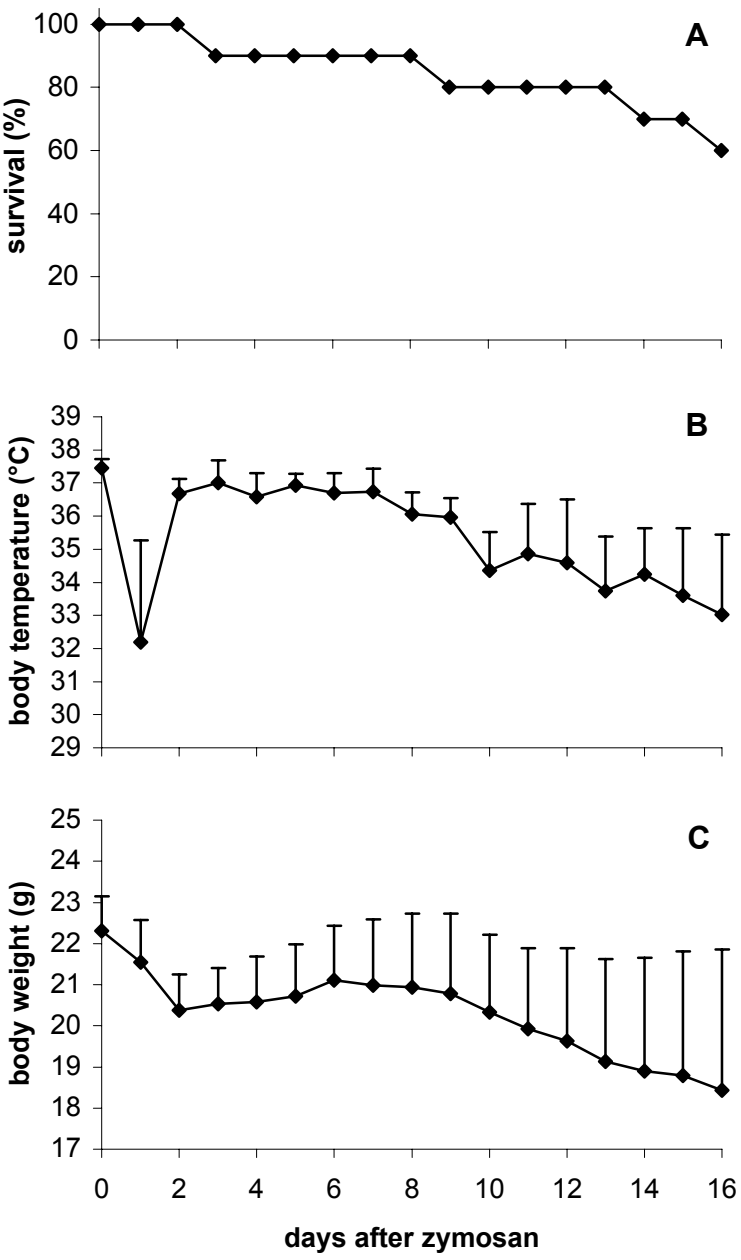

Figure 1. Survival and clinical symptoms after zymosan administration. Panel A depicts survival; Body temperature (B) and body weight (C) were measured daily and are depicted as average + SD in a group which comprised 10 animals at the start of the experiment.

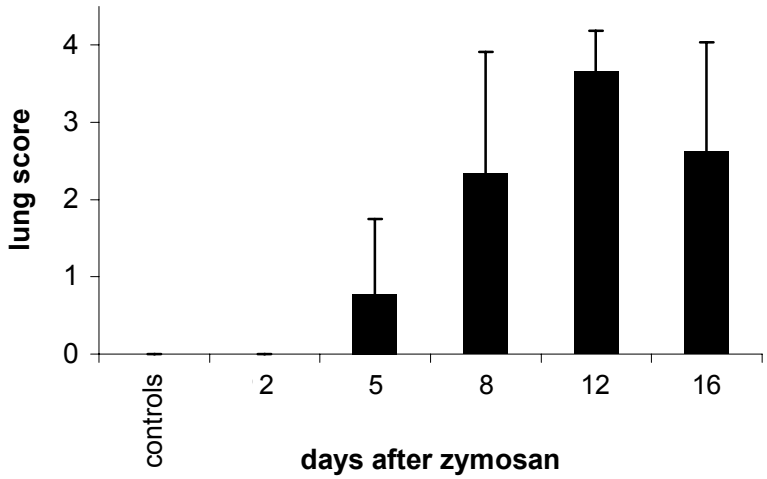

Figure 2. Development of lung damage. Bars represent average values + SD. (n=9, except for day 12 where n=6) for the macroscopic lung score; 0: no hemorrhages 1: hemorrhagic spots covering approximately 1-25% of the lung surface; 2: confluent hemorrhagic spots covering approximately 25-50% of the lung surface; 3: large areas of hemorrhage covering 50-75% of the lung surface; 4: completely hemorrhagic lungs (75-100% of the surface covered with hemorrhages).

higher than in controls at day 5 (Figure 3B). In the spleen and kidneys, the mRNA expression of MMP-2 was upregulated only marginally while the increase in MMP-9 mRNA was much more pronounced (Figure 3C and 3D).

Bioactivity

Quantitative gelatin zymography demonstrated the near complete absence of any gelatinase activity in tissues from control mice. However, in the liver of zymosan-treated animals, a significantly increased activity of proform (60 kDa) and active (53 kDa) MMP-2 and of proform (100-108 kDa) and active (80-92 kDa) MMP-9 was observed with time. This was especially the case for proMMP-9, which showed a strong and lasting elevation from day 5 onwards (Figure 5A). The same was true for the lungs (Figure 5B). The highest

gelatinase activity was observed in the spleen (Figure 5C). In contrast to the other tissues, the spleen also exhibited very high levels of active MMP-9, peaking around day 8.

In the kidneys, only low levels of gelatinase activity were observed at any time point (data not shown).

Next to the specific bands of proform and active MMP-2 and -9, the zymograms contained other gelatinase activities, especially at higher kDa values, which represent dimeric forms or complexes between MMPs and inhibitors. All activities were inhibited by EDTA (data not shown). For each sample, we have also calculated the total gelatinase activity on each zymogram. Figure 6 illustrates that the total gelatinase activity was strongly increased during the development of organ failure.

In addition to the gelatinase activities, we also measured collagenase bioactivity in a selected number of samples. Overall, MMP-1 and –13 activities were very low in all samples from liver and lung (Figures 7A and 7B, respectively). In the liver, the MMP-13 activity at day 8 was significantly lower than in controls. In the lungs, both MMP-1 and –13 activities were significantly lower in the zymosan-treated mice than in the control animals. Much higher levels of MMP-1 activity were found in the spleen from zymosan-treated mice (Figure 7C). MMP-13 activities in were much lower, but still significantly higher than in the untreated control animals.

Immunohistochemistry

Immunohistochemical staining for MMP-2 and MMP-9 could be observed in organs of mice that had been killed at the various time points in the model. However, because of the great variability in staining between animals and the relative small number of animals per time point, it was not possible to quantify

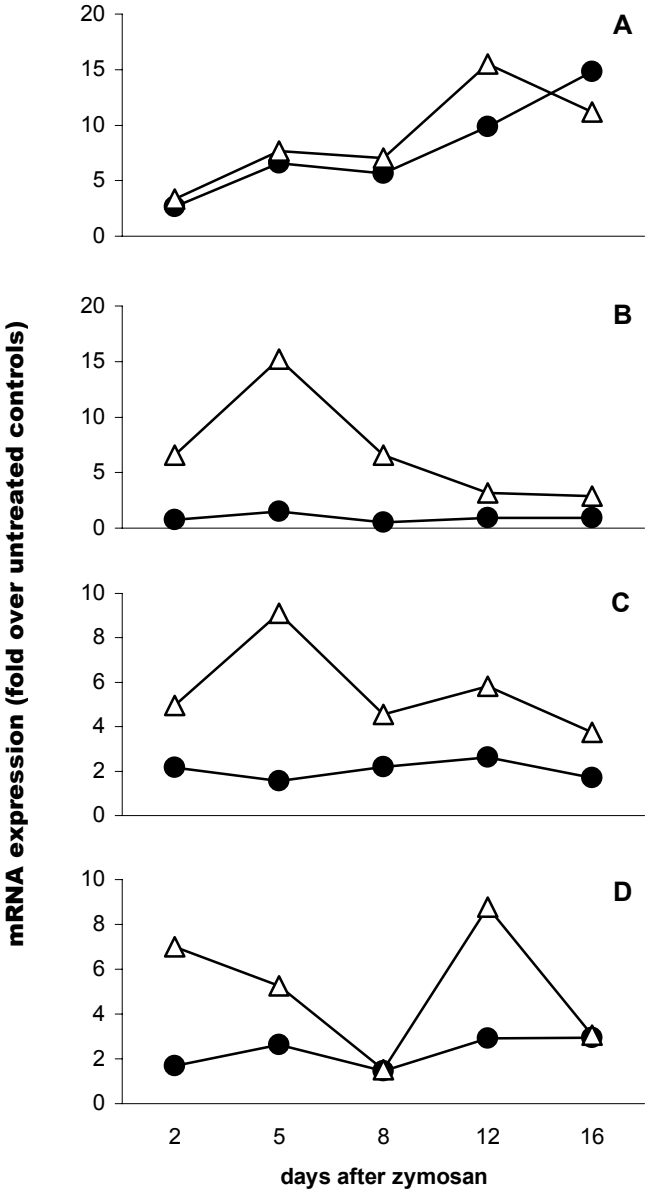

Figure 3. Upregulation of MMP-2 (●) and MMP-9 (Δ) mRNA in liver (A), lung (B), spleen (C) and kidney (D) tissues, quantitated by a real-time RT-PCR. The degree of upregulation was calculated for each animal as the mRNA expression at any time point in relation to the median mRNA expression in control mice. Data are represented as medians (n=6).

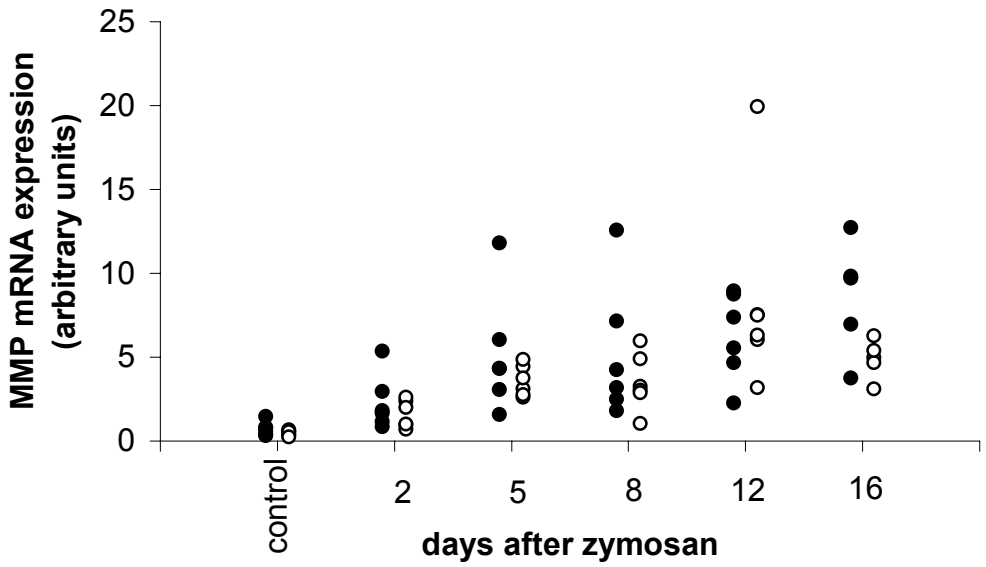

Figure 4. mRNA expression of MMP-2 (●) and MMP-9 (o) in liver tissue of individual mice, quantitated by a real-time RT-PCR.

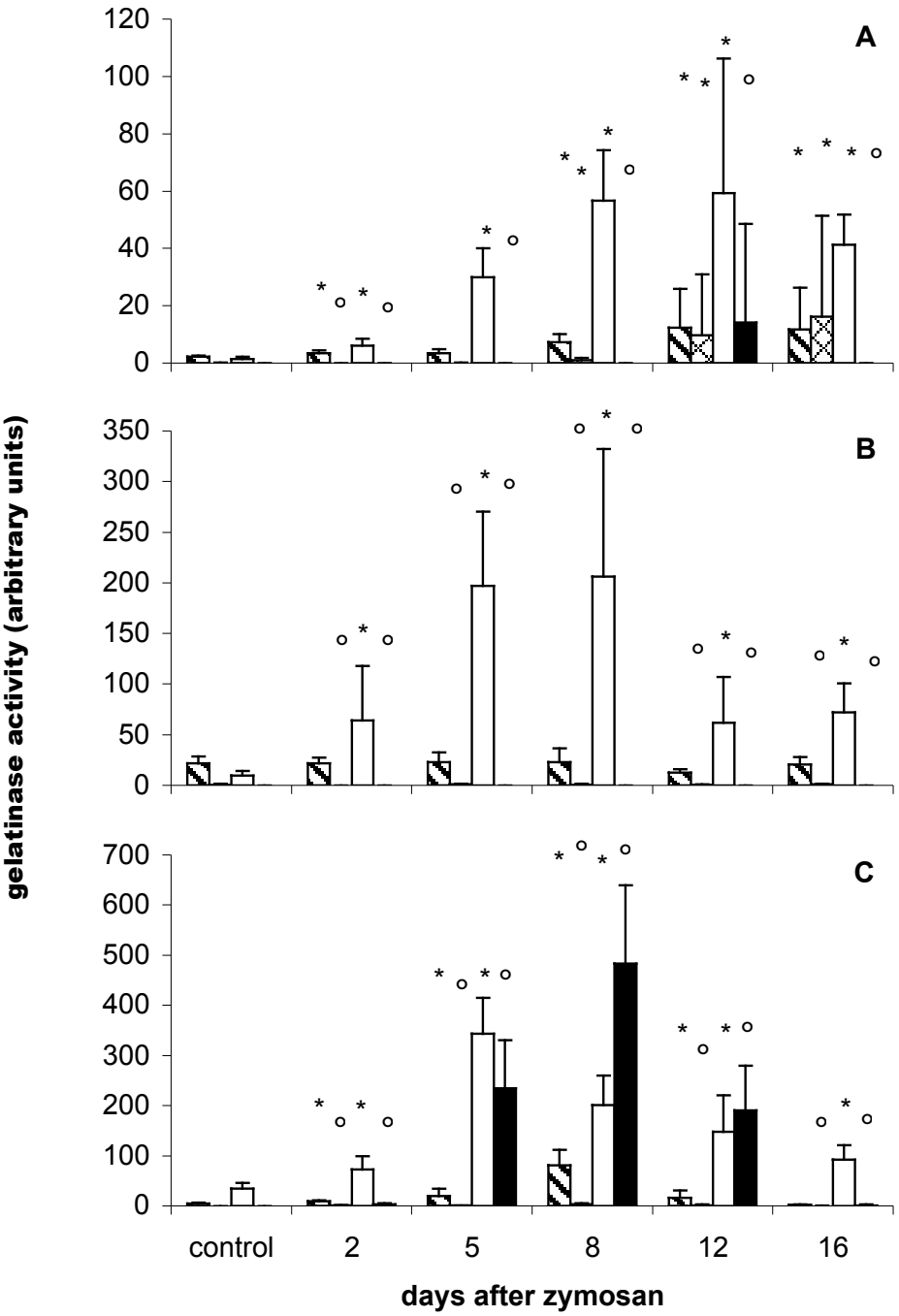

Figure 5. Tissue MMP-2 and MMP-9 activities. The activity of proMMP-2 (hatched bars), active MMP-2 (double-hatched bars), proMMP-9 (open bars) and active MMP-9 (closed bars) in liver (A), lung (B) and spleen (C) extracts were quantitated by gelatin zymography. Data represent specific activity in arbitrary units/mg protein (average + SD, n=6). Bars marked with an asterisk are significantly different from control animals (P<0.05, Mann-Whitney U test). Bars marked with a circle indicate that testing was not possible due to an SD of zero in one or more groups.

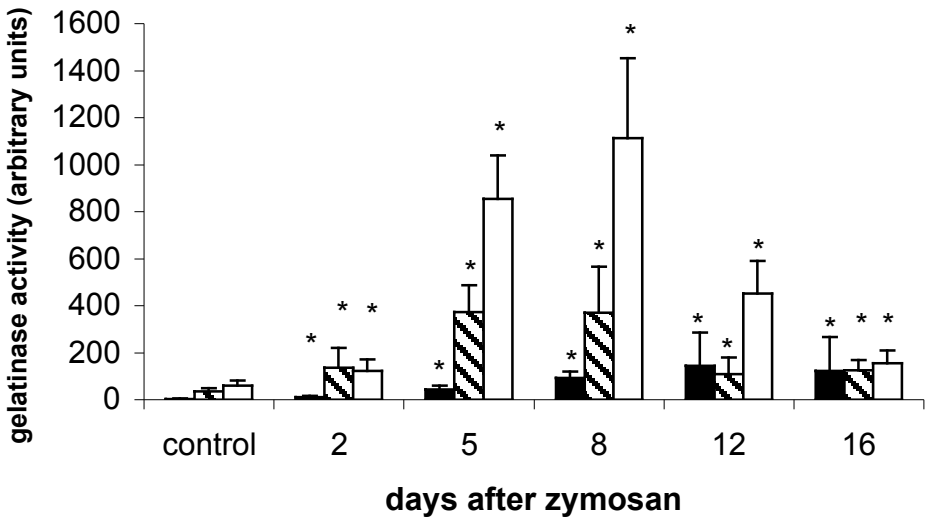

Figure 6. Total tissue gelatinase activity. Bars represent average (+ SD, n=6) total gelatinolytic activity measured on zymograms from liver (closed bars), lung (hatched bars) and spleen (open bars). Bars marked with an asterisk are significantly different from control animals (P<0.05, Mann-Whitney U test).

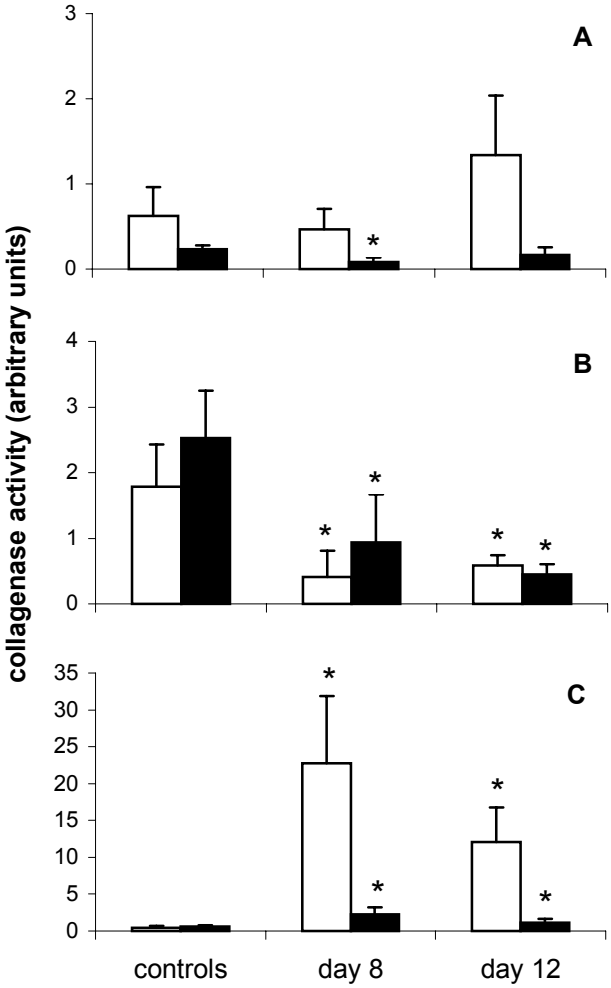

Figure 7. Tissue MMP-1 and MMP-13 activities. The activity of MMP-1 (open bars) and MMP-13 (closed bars) in liver (A), lung (B) and spleen (C) extracts was quantitated by fluorogenic assays. Data represent specific activity in arbitrary units/mg protein (average + SD, n=5). Bars marked with an asterisk are significantly different from control animals (P<0.05, Mann-Whitney U test).

any changes in the expression of these MMPs over time during the course of the experiment.

MMP-2 was detected in zymosan-treated animals at all time points, i.e. in the granulomatous lesions of the liver and in the red pulp of the spleen but not in lung and kidney tissue of zymosan-treated mice. In untreated animals all tissues were negative. Staining appeared to be limited to phagocyting, macrophage-like cells (Figure 8).

MMP-9 could be detected in liver, lung and spleen tissues of zymosan-treated mice at all time points. In untreated control animals, organs were negative except for the spleen. Here, a slight degree of staining for MMP-9 was observed although the reaction in spleen tissues from zymosan-treated mice was much stronger. As for MMP-2, staining for MMP-9 was limited to phagocytes (Figure 9).

DISCUSSION

The data demonstrate that there is an upregulation of gelatinase mRNA and an increased MMP activity in multiple tissues during the development of MODS in mice. Organ damage in this model is accompanied by gradual and progressive changes in wet and dry organ weights that correlate with increasing pathology. Histopathological changes include progressive development of massive hemorrhagic spots in the lungs and granuloma-like structures of mononuclear cells in the liver. In the spleen, the distinct borders between red and white pulp disappear and there are megakaryocytes and lymphocytes present in the red pulp [5].

In general, MMPs are not produced constitutively. Their expression is regulated in a cell-specific manner by inflammatory mediators such as growth factors and proinflammatory cytokines [25]. Of the MMPs examined in the current study, the expression of MMP-9 was increased most strongly. MMP-9 attacks basement

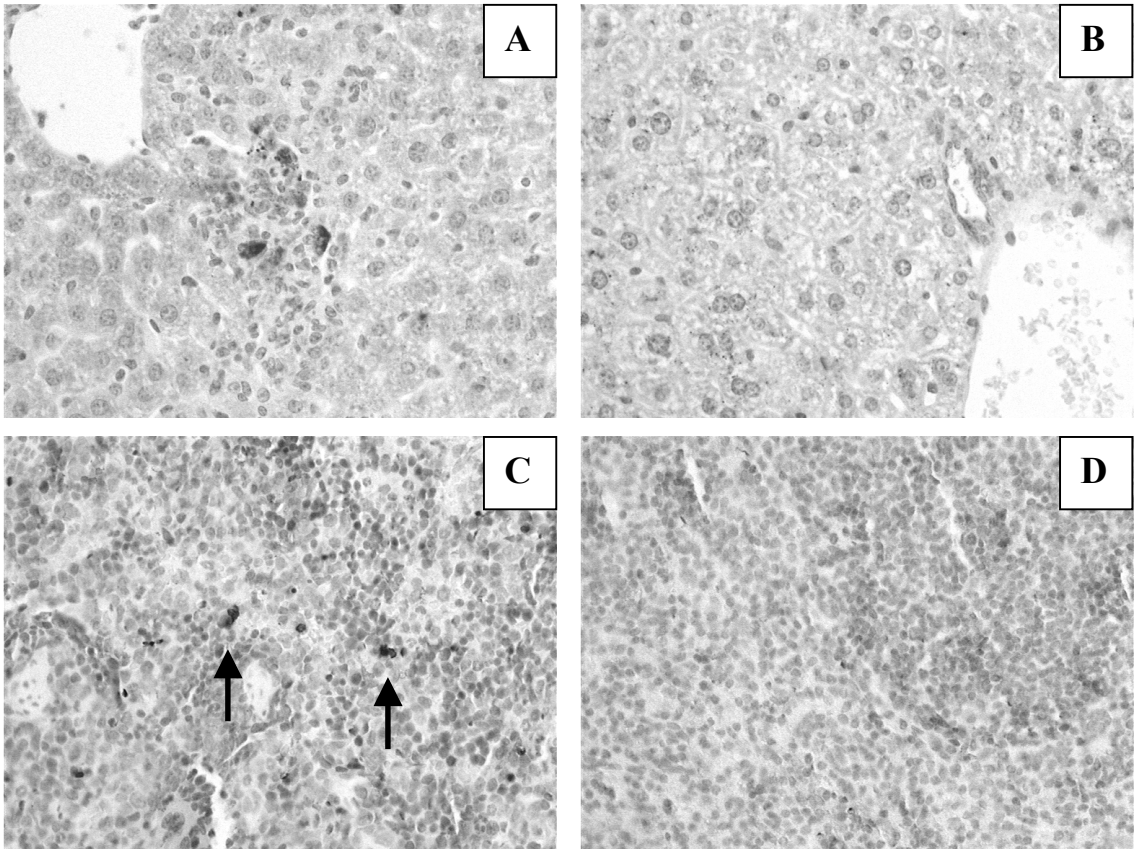

Figure 8. Immunohistochemical staining for MMP-2. Staining was observed in phagocytes in liver (A) and spleen (C; arrows) of zymosan-treated mice, but absent in liver (B) and spleen tissues (D) of untreated control mice.

membrane collagen, elastin, and fibronectin, and also efficiently degrades denatured collagens or gelatins of all types [19]. It is secreted as a zymogen and processed to an active form via a conformational change at the catalytic site, followed by autocatalysis [26]. PMNs and endothelial cells contain a stock of preformed MMP-9 in secretory vesicles [20,27], which enables them to release MMP-9 very rapidly following specific stimuli. Although proMMP-9 can be secreted by many cell types involved in the inflammatory response, the immunohistochemical data indicate that in the present model MMP-9 is produced by phagocytic cells, probably macrophages.

MMP-9 has been shown to cleave inactive TNF-α, such that active TNF-α is released [28]. In addition, MMP-9 gene expression by macrophages and PMNs and

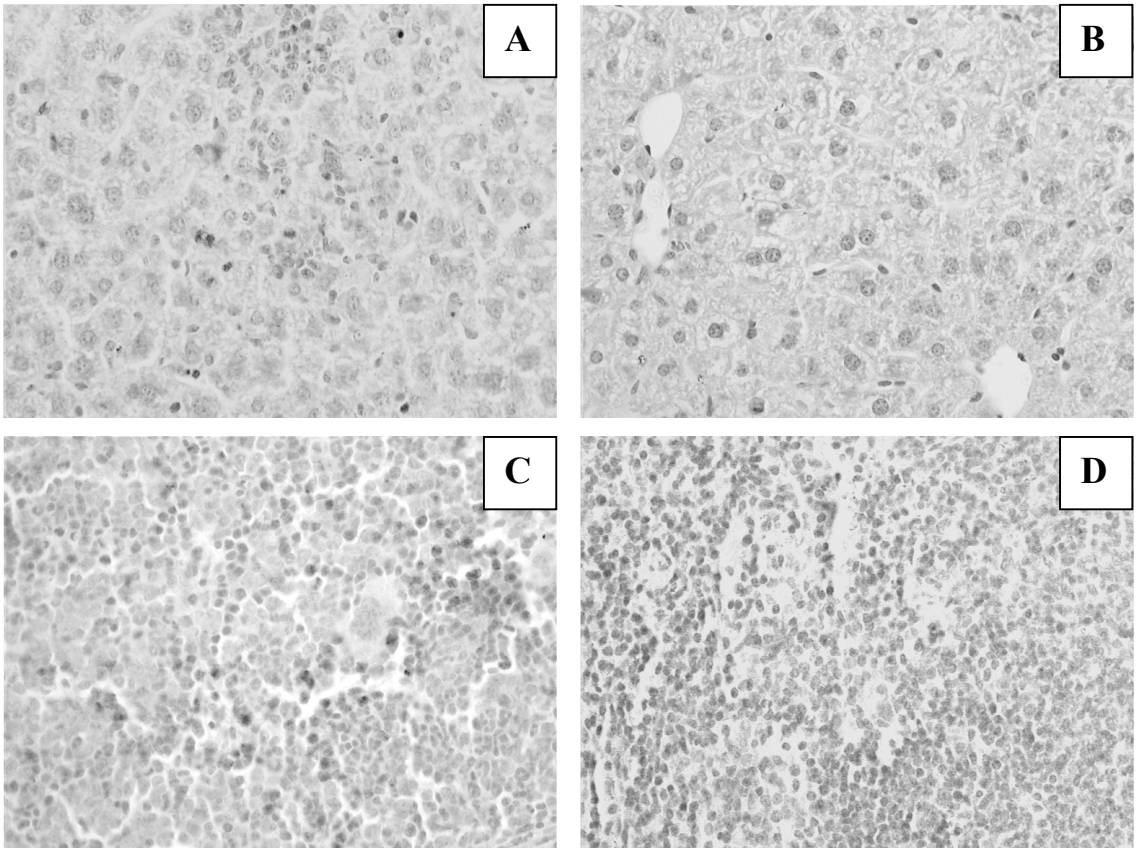

Figure 9. Immunohistochemical staining for MMP-9. Staining was observed in phagocytes in liver (A) and spleen (C) tissues of zymosan-treated mice. Liver of untreated control mice (B) was negative. Slightly stained cells were present in the red pulp of the spleen (D).

hence the release of proMMP-9 can be induced by TNF-α and IL-1β [19,20]. Tissue inhibitor of metalloproteinase-1 (TIMP-1) production by macrophages is not affected by these cytokines [19], suggesting that an excess of MMP-9 molecules due to a rise in TNF-α and IL-1β concentrations remains catalytically competent.

It has been extensively reported that TNF-α levels are strongly elevated in critically ill patients [16]. Also, previous studies in our laboratory have shown that in the third - MODS-like - phase of the murine model mRNA levels for TNF-α and IL-1β are elevated in several organs, as are circulating TNF-α levels. It is conceivable that elevated levels of TNF-α and/or IL-1β are responsible for the increased activity of MMP-9 observed.

We also observed an increased expression and activity of MMP-2 in the course of the model, but this increase is weaker than for MMP-9 and remains limited to spleen and liver tissue. Although MMP-2 has a substrate specificity which is similar to MMP-9, its expression is regulated differently [27]. ProMMP-2 is constitutively expressed by many cell types [29], but its expression is also induced by specific stimuli [30]. Unlike MMP-9, MMP-2 is not induced by TNF-α and IL-1β. Next to its gelatinase activity, MMP-2 cleaves proMMP-9, yielding active MMP-9 [30].

Basement membrane is the part of the extracellular matrix that is associated with the vascular endothelium. It is also associated with hepatocytes and bile duct epithelium in the liver, the alveolar epithelium in the lungs and epithelial cells and glomeruli in the kidneys. An increased activity of MMP-2 and –9 might threaten the integrity of basement membranes and may thereby lead to capillary leakage, increased influx of inflammatory cells in tissues and organ damage.

The activity of the collagenases MMP-1 and MMP-13 is also increased in spleen tissue at days 8 and 12 after zymosan. Since phagocytosis of zymosan results in the production of MMP-1 by macrophages [31], it is possible that macrophages are responsible for the increased production of MMP-1. Apart from its collagenase activity, MMP-1 activates proMMP-2 and -9 [30], thereby possibly contributing to increased gelatinase activity. The activities of MMP-1 and –13 in liver and lung tissues in zymosan-treated animals are significantly lower than in control animals in most cases, but since MMP-1 and –13 activities in these organs are very low to start with, this difference is probably not of any clinical relevance.

The results from PCR and zymography show that gelatinase mRNA expression and activity in the lungs and spleen peak at days 5 to 8, whereas in the liver peak expression and activity are reached later, at days 12 to 16. This is in agreement with the occurrence of organ damage during the development of MODS in mice, because in earlier studies we have observed that lung damage precedes liver damage [5].

MMPs have been studied in relation to generalized inflammation in mice before. Pagenstecher et al. [32] have reported an increased expression of MMP genes during LPS-induced endotoxemia in mice, suggesting that MMPs may contribute to the development of organ damage in endotoxemia.

The results from the present study imply that administration of MMP inhibitors might be beneficial in this MODS model. MMP inhibitors have been studied in other models of critical illness. Carney et al. [33] have found that pretreatment of pigs with COL-3, a combined MMP and elastase inhibitor, results in lower levels of MMP-2 and –9 in bronchoalveolar lavage fluid and a lesser degree of organ injury in an ARDS model. Another MMP inhibitor, GM-6001, has been shown to improve survival in an endotoxic shock model in mice, probably by inhibiting TNF-α processing and release [34].

In conclusion, our study suggests a role for MMPs, especially MMP-9, in the pathogenesis of MODS. Intervention studies with MMP inhibitors could provide additional evidence for this hypothesis. If intervention studies with animals show promising results, MMP inhibitors might eventually provide a clinical treatment option for patients who are at risk for MODS.

ACKNOWLEDGMENTS

We thank Ben de Man for his expert technical assistance.

References

1. Seely AJ, Christou NV. Multiple organ dysfunction syndrome: exploring the paradigm of complex nonlinear systems. Crit Care Med 2000; 28:2193-2200.

2. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit Care Med 2001; 29:S99-106.

3. Goris RJ, Boekholtz WK, van Bebber IP, Nuytinck JK, Schillings PH. Multiple-organ failure and sepsis without bacteria. An experimental model. Arch Surg 1986; 121:897-901.

4. Goris RJ. MODS/SIRS: result of an overwhelming inflammatory response? World J Surg 1996; 20:418-421.

5. Jansen MJ, Hendriks T, Verhofstad AA, Lange W, Geeraedts-LM J, Goris RJ. Gradual development of organ damage in the murine zymosan-induced multiple organ dysfunction syndrome. Shock 1997; 8:261-267.

6. Volman TJH, Goris RJ, van der Jagt M, van de Loo FAJ, Hendriks T. Organ damage in zymosan-induced multiple organ dysfunction syndrome in mice is not mediated by inducible nitric oxide synthase. Crit Care Med 2002; 30:1553-1559.

7. Demling R, Lalonde C, Youn YK, Daryani R, Campbell C, Knox J. Lung oxidant changes after zymosan peritonitis: relationship between physiologic and biochemical changes. Am Rev Respir Dis 1992; 146:1272-1278.

8. Shayevitz JR, Miller C, Johnson KJ, Rodriguez JL. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. Shock 1995; 4:389-396.

9. Cuzzocrea S, McDonald MC, Mazzon E, Filipe HM, Centorrino T, Lepore V, Terranova ML, Ciccolo A, Caputi AP, Thiemermann C. Beneficial effects of tempol, a membrane-permeable radical scavenger, on the multiple organ failure induced by zymosan in the rat. Crit Care Med 2001; 29:102-111.

10. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 2003; 200:448-464.

11. Itoh T, Matsuda H, Tanioka M, Kuwabara K, Itohara S, Suzuki R. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. J Immunol 2002; 169:2643-2647.

12. Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001; 44:585-594.

13. George SJ. Therapeutic potential of matrix metalloproteinase inhibitors in atherosclerosis. Expert Opin Investig Drugs 2000; 9:993-1007.

14. Ricou B, Nicod L, Lacraz S, Welgus HG, Suter PM, Dayer JM. Matrix metalloproteinases and TIMP in acute respiratory distress syndrome. Am J Respir Crit Care Med 1996; 154:346-352.

15. Torii K, Iida K, Miyazaki Y, Saga S, Kondoh Y, Taniguchi H, Taki F, Takagi K, Matsuyama M, Suzuki R. Higher concentrations of matrix metalloproteinases in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Am J Respir Crit Care Med 1997; 155:43-46.

16. Yassen KA, Galley HF, Webster NR. Matrix metalloproteinase-9 concentrations in critically ill patients. Anaesthesia 2001; 56:729-732.

17. Jansen MJ, Hendriks T, Vogels MT, Van der Meer JW, Goris RJ. Inflammatory cytokines in an experimental model for the multiple organ dysfunction syndrome. Crit Care Med 1996; 24:1196-1202.

18. Volman TJH, Hendriks T, Verhofstad AA, Kullberg BJ, Goris RJ. Improved survival of TNF-deficient mice during the zymosan-induced multiple organ dysfunction syndrome. Shock 2002; 17:468-472.

19. Saren P, Welgus HG, Kovanen PT. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. J Immunol 1996; 157:4159-4165.

20. Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HL, Suffredini AF. Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. Am J Respir Cell Mol Biol 1999; 20:458-464.

21. Waas ET, Lomme RM, DeGroot J, Wobbes T, Hendriks T. Tissue levels of active matrix metalloproteinase-2 and -9 in colorectal cancer. Br J Cancer 2002; 86:1876-1883.

22. Drijfhout J, Nagel J, Beekman B, TeKoppele J, Bloemhoff W. Solid phase synthesis of peptides containing the fluorescence energy transfer Dabcyl-Edans couple. In Kaumaya P, Hodges R (eds). Peptides. Chemistry, Structure and Biology. Mayflower Sci. Ltd., United Kingdom; 1996:129-131.

23. DeGroot J, Verzijl N, Budde M, Bijlsma JW, Lafeber FP, TeKoppele JM. Accumulation of advanced glycation end products decreases collagen turnover by bovine chondrocytes. Exp Cell Res 2001; 266:303-310.

24. Marijnissen AC, van Roermund PM, Verzijl N, TeKoppele JM, Bijlsma JW, Lafeber FP. Steady progression of osteoarthritic features in the canine groove model. Osteoarthritis Cartilage 2002; 10:282-289.

25. Nagase H, Woessner J-FJ. Matrix metalloproteinases. J Biol Chem 1999; 274:21491-21494.

26. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Chem Biol 1996; 3:895-904.

27. Nguyen M, Arkell J, Jackson CJ. Human endothelial gelatinases and angiogenesis. Int J Biochem Cell Biol 2001; 33:960-970.

28. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, et a. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 1994; 370:555-557.

29. Lanchou J, Corbel M, Tanguy M, Germain N, Boichot E, Theret N, Clement B, Lagente V, Malledant Y. Imbalance between matrix metalloproteinases (MMP-9 and MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in acute respiratory distress syndrome patients. Crit Care Med 2003; 31:536-542.

30. Johnson LL, Dyer R, Hupe DJ. Matrix metalloproteinases. Curr Opin Chem Biol 1998; 2:466-471.

31. Shapiro SD, Kobayashi DK, Welgus HG. Identification of TIMP-2 in human alveolar macrophages. Regulation of biosynthesis is opposite to that of metalloproteinases and TIMP-1. J Biol Chem 1992; 267:13890-13894.

32. Pagenstecher A, Stalder AK, Kincaid CL, Volk B, Campbell IL. Regulation of matrix metalloproteinases and their inhibitor genes in lipopolysaccharide-induced endotoxemia in mice. Am J Pathol 2000; 157:197-210.

33. Carney DE, McCann UG, Schiller HJ, Gatto LA, Steinberg J, Picone AL, Nieman GF. Metalloproteinase inhibition prevents acute respiratory distress syndrome. J Surg Res 2001; 99:245-252.

34. Solorzano CC, Ksontini R, Pruitt JH, Auffenberg T, Tannahill C, Galardy RE, Schultz GP, MacKay SL, Copeland EM, Moldawer LL. A matrix metalloproteinase inhibitor prevents processing of tumor necrosis factor alpha (TNF alpha) and abrogates endotoxin-induced lethality. Shock 1997; 7:427-431.

SUMMARY / SAMENVATTING

SUMMARY

Unfortunately, our increased ability to keep critically ill patients alive has yielded a new phenomenon: the multiple organ dysfunction syndrome (MODS). MODS is defined as the presence of altered organ function in a severely ill patient so that homeostasis cannot be maintained without intervention. Regardless of the cause, MODS typically consists of the sequential dysfunction of several organ systems, beginning with the lungs and followed by hepatic, intestinal, renal, and eventually cardial dysfunction, although the exact order may vary because of preexisting disease or the nature of the precipitating insult. MODS usually occurs after various physiologic insults, which may include pancreatitis, trauma, burns, shock, severe infection, aspiration, multiple blood transfusions, and pulmonary contusions. There are two clinical observations that strongly indicate that MODS is a systemic process. First, the organs where damage occurs are not necessarily directly injured or involved in the primary disease process. Second, there is usually a lag phase of days to weeks between the primary insult and the development of remote organ dysfunction. These observations suggest that endogenous circulating factors are involved in the pathophysiology of MODS. Studies in critically ill patients show that increased circulating levels of pro-inflammatory cytokines are associated with organ dysfunction and an increased risk of death. It is now generally believed that MODS is the result of a generalized inflammatory response (chapter 1).
The examination of the mechanisms behind MODS requires invasive procedures that cannot be performed on humans. Therefore, animal studies and *in vitro* studies are needed to elucidate the pathophysiological pathways that lead to MODS. In our laboratory, an animal model for MODS has been developed. It is called the zymosan-induced generalized inflammation (ZIGI) model and has been adopted by other research groups as well (chapter 2).

Intraperitoneal injection of zymosan in C57BL/6 mice leads to a three-phasic illness starting with a peritonitis-like reaction. This first phase lasts for 2 days and renders the animals very ill, which is apparent from a transient decrease in body temperature, loss of body weight, and a mortality of up to 20%. After 2 days the surviving mice appear to recover, indicated by a normalization of body temperature and weight. After about 7 days, the third phase sets in. This phase is characterized by MODS-like symptoms, i.e. cachexia, tachypnea and hypothermia. In addition, progressive changes in the morphology of the lungs, liver, spleen and kidneys occur, also reflected by an increase in organ weight. Hemorrhagic spots are found in the lungs evolving into macroscopically visible massive hemorrhages, a process that can be quantitated by using a lung score. An additional 20-30% of the animals die in this phase before the experiment is terminated after 12 to 22 days.

A lot of research has focussed on identifying the mediators involved in the development of MODS. Circulating inflammatory mediators are relatively easy to measure, but may be a poor reflection of processes at organ level, where the damage occurs. To devise logical interventions, possibly directed at multiple cytokines, it is necessary to collect data on their expression at tissue level during the development of MODS. For this purpose, we have performed a comprehensive study quantitating the course of TNF-α, IL-1β, IL-6, MIF, IL-12, IFN-γ and IL-10 mRNA expression in lung, liver, spleen and kidney, tissues that exhibit increasing histopathological changes in time (chapter 3). During the peritonitis phase, upregulation of cytokine mRNA was limited. During the period of apparent recovery, cytokine mRNA expression strongly increased, mostly reaching its maximum at day 9 when deterioration of the clinical condition had already set in. The upregulation of TNF-α mRNA was most pronounced, especially in the lungs and liver. Thus, interventions should preferentially be targeted against multiple cytokines and, at least in this model, there may be a treatment window well after the initial challenge.

Previous research in our laboratory has shown that plasma TNF-α levels are increased during the first 24 h after injection of zymosan, followed by a prominent peak from day 8 onwards. Moreover, subsequent experiments have revealed that interventions directed at TNF-α may mitigate the symptoms of zymosan-induced MODS, indicating that TNF-α plays a prominent role in the pathophysiology. Additional evidence for this hypothesis is provided by experiments using TNF knockout mice. The complete absence of TNF results in a significantly lower mortality in the ZIGI model (chapter 4). In addition, TNF knockout mice exhibit a higher average body temperature and average body weight than wildtype mice at day 22. However, at this time point, surviving animals from both groups show similar and significant microscopic damage in lungs, liver and spleen and an elevated average organ weight. Thus, although TNF-deficient mice exhibit significantly improved morbidity and mortality, the absence of TNF does not completely protect against zymosan-induced MODS. Since strategies directed at counteracting TNF-α alone have so far only been partially successful in preventing MODS in the murine model, targeting multiple cytokines at the same time appears likely to have more beneficial effects.. Pentoxifylline (PTX) is a methylxanthine derivative that has been used for treatment of chronic occlusive arterial disease because of its rheological actions. PTX has effects on the inflammatory response as well. It has been shown to decrease TNF-α, IL-1β and IL-6 levels in sepsis models. PTX is known to inhibit phosphodiesterase, thereby increasing cytoplasmic cyclic adenosine monophosphate levels. This strongly inhibits TNF-α gene transcription. Because of its ability to counteract multiple pro-inflammatory mediators simultaneously, PTX is a potentially useful agent to attenuate the generalized inflammatory response leading to the organ failure observed in MODS. We have performed an intervention study to investigate the effects of PTX administration (chapter 5). *Ex vivo*, PTX inhibits the LPS-induced TNF-α and IL-6 production (but not the IL-1β production) in J774.A1 macrophages in a dose-dependent manner, both at

mRNA and protein level. Administration of PTX to zymosan-treated C57BL/6 mice does not result in a significantly improved survival, body temperature, body weight or lung score. Also, no significant differences in organ weights have been found between mice that received PTX and mice that received PBS. TNF-α and IL-6 mRNA expression in the liver is similar in both groups and this is probably the main reason why no beneficial effects of PTX on morbidity and mortality have been observed. Altogether, these results do not support the hypothesis that PTX has beneficial effects in zymosan-induced organ damage in mice.

During inflammation and shock, proinflammatory cytokines and bacterial wall components may induce nitric oxide synthase (iNOS). NO produced by iNOS has been proposed to cause tissue damage, either directly or indirectly by the formation of peroxynitrite. We have investigated the role of iNOS in the development of murine MODS using either a selective iNOS inhibitor (aminoguanidine) or iNOS knockout mice (chapter 6). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry reveal a strongly increased expression of iNOS messenger RNA (mRNA) and iNOS protein in the livers of C57BL/6 mice in the third – MODS-like - phase of the model. However, neither the *in vivo* administration of aminoguanidine to C57BL/6 mice nor the complete absence of iNOS enzyme (iNOS knockout mice) have a beneficial effect on survival, body temperature or body weight. In addition, lung, liver, and spleen weights and lung scores are similar in the various experimental groups. These results strongly argue against an essential and causative role of iNOS in the development of murine MODS.

The extracellular matrix is central to tissue architecture and homeostasis. Disruption of tissue architecture will result in organ dysfunction. Remodelling of the extracellular matrix, either as part of physiological homeostasis and development or as part of a pathological process, is to a large part mediated by enzymes from the matrix metalloproteinase (MMP) family, which consists of a

growing number of secreted and membrane-bound metalloendopeptidases. During inflammation, they play an important role in the control of cellular interactions with their environment. MMPs have been implicated as mediators of tissue damage in several inflammatory diseases. MMPs have also been related to systemic inflammation. Several studies have suggested an important role for MMPs in the development of the adult respiratory distress syndrome (ARDS) and it has been shown recently that circulating MMP-9 concentrations are elevated in critically ill patients. It seems entirely possible that an overactivation of MMPs could contribute to the organ damage observed in MODS. Gene expression of MMPs is regulated by several factors, among them cytokines. An experiment has been conducted to provide data on the expression and activity of several gelatinases and collagenases in lungs, liver, spleen and kidneys during zymosan-induced MODS (chapter 7). The expression of MMP-2 mRNA in zymosan-treated mice is strongly upregulated in liver tissue only. For MMP-9, this is the case in all organs examined. In the liver, lungs and especially the spleen of zymosan-treated animals, a significantly increased activity of proform and active MMP-2 and –9 is observed with time. MMP-1 and –13 activities are very low in all samples from liver and lung. In the spleen, however, high levels of MMP-1 and –13 are observed in zymosan-treated animals.
Immunohistochemical staining for MMP-2 is detected in the liver and the spleen but not in lung and kidney tissue of zymosan-treated animals. Specific staining for MMP-9 is present in liver, lung and spleen tissue of zymosan-treated mice. For both MMPs, staining appears to be limited to phagocytes. These results suggest a role for MMPs, especially MMP-9, in the pathogenesis of MODS. Intervention studies with MMP inhibitors could provide additional evidence for this hypothesis.
Since MODS does not seem to be driven by a single mediator, interventions directed at multiple mediators seem the most promising approach for the future. A major problem will be finding or designing agents that can modulate multiple

mediators in a specific way at a specific time point of illness. Previous studies directed at multiple mediators have been performed using treatments that were not specific while the mechanisms of actions were often not entirely clear, making interpretations of the results difficult. Hopefully, specific interventions such as inhibiting multiple proinflammatory cytokines simultaneously, without intervening with other cellular processes, will become possible in the near future. This will improve our understanding of the underlying pathophysiology of MODS and will eventually provide better treatment options in patients at risk for or with MODS.

SAMENVATTING

Onze toegenomen mogelijkheden om patiënten die zich in een kritieke toestand bevinden in leven te houden heeft helaas een nieuw fenomeen opgeleverd: het multi-orgaan disfunctie syndroom (MODS). MODS wordt gedefinieerd als een zodanige verslechtering van orgaanfuncties in een ernstig zieke patiënt dat de homeostase zonder interventie niet gehandhaafd kan worden. MODS kenmerkt zich door het achtereenvolgens ontregelen van verschillende orgaansystemen, meestal beginnend met de longen. Dit wordt gevolgd door functieverlies van de lever, het darmstelsel, de nieren en uiteindelijk het hart, hoewel de exacte volgorde kan variëren onder invloed van onderliggende ziekten of de aard van het voorafgaande insult. MODS kan optreden na diverse fysiologische insulten, onder andere pancreatitis, trauma, brandwonden, shock, ernstige infecties, meervoudige bloedtransfusies en longcontusies. Er zijn twee klinische observaties die sterk suggereren dat MODS een systemisch proces is. Ten eerste zijn de organen waar schade optreedt niet noodzakelijkerwijs betrokken bij het primaire ziekteproces. Ten tweede is er vaak een tijdsinterval van dagen tot weken tussen het primaire insult en het ontstaan van orgaandisfunctie elders in het lichaam. Deze waarnemingen suggereren dat endogene factoren in de circulatie betrokken zijn bij de pathofysiologie van MODS. Studies bij patiënten in kritieke toestand laten zien dat een toegenomen concentratie van proinflammatoire cytokinen samenhangt met orgaanfalen en een verhoogde kans op overlijden. Het wordt nu algemeen aanvaard dat MODS het resultaat is van een gegeneraliseerde ontstekingsreactie (hoofdstuk 1).

Onderzoek naar de onderliggende mechanismen bij MODS vereist invasieve procedures die niet uitgevoerd kunnen worden bij mensen. Om opheldering te krijgen over de pathofysiologie zijn daarom dierexperimenten en *in vitro* studies noodzakelijk. In ons laboratorium is een diermodel voor MODS ontwikkeld. Het

wordt het *zymosan-induced generalized inflammation* (ZIGI) model genoemd en is ook overgenomen door andere onderzoeksgroepen (hoofdstuk 2).
Een intraperitoneale injectie van zymosan in C57BL/6 muizen leidt tot een drie-fasisch ziektebeeld, beginnend met een peritonis-achtige reactie. In deze eerste fase, die twee dagen duurt, zijn de dieren erg ziek, hetgeen blijkt uit een tijdelijke afname in lichaamstemperatuur, een afname van het lichaamsgewicht, en een mortaliteit van maximaal 20%. Na 2 dagen lijken de overlevende muizen te herstellen, de lichaamstemperatuur en het lichaamsgewicht normaliseren weer. Na ongeveer 7 dagen begint de derde fase. Deze fase wordt gekarakteriseerd door MODS-achtige signalen, namelijk cachexie, tachypnoe en hypothermie. Tevens treden progressieve veranderingen in de morfologie van de longen, lever, milt en nieren op, die ook weerspiegeld worden door een toename van de orgaangewichten. In de longen treden bloedingen op die zich ontwikkelen tot grote, macroscopisch zichtbare, bloeduitstortingen. Dit proces kan gekwantificeerd worden door gebruik te maken van een longscore. In deze fase sterven nog eens 20-30% van de dieren, alvorens het experiment na 12 tot 22 dagen beëindigd wordt.
Veel onderzoek heeft zich gericht op het identificeren van de mediatoren die betrokken zijn bij het ontstaan van MODS. Ontstekingsmediatoren in de circulatie zijn relatief gemakkelijk te meten, maar het is de vraag in hoeverre deze metingen een juiste weerspiegeling zijn van processen op orgaanniveau, waar de schade optreedt. Om logische interventies te bedenken, die mogelijk gericht zijn tegen meerdere cytokines, is het nodig om data te verzamelen over de expressie van deze cytokines op orgaanniveau gedurende het ontstaan van MODS. Daarom hebben we een uitgebreide studie verricht waarin we het beloop van de expressie van TNF-α, IL-1β, IL-6, MIF, IL-12, IFN-γ en IL-10 mRNA onderzocht hebben in de longen, lever, milt en nieren (hoofdstuk 3). Dit zijn weefsels die in de loop van de tijd een toenemende mate van histopathologische veranderingen laten zien. Tijdens de peritonitisfase bleef de opregulatie van

cytokine mRNA beperkt. Tijdens de periode van ogenschijnlijk herstel nam de expressie van cytokine mRNA sterk toe, waarbij het maximum meestal bereikt werd op dag 9, toen de verslechtering van de klinische toestand al was begonnen. De opregulering van TNF-α mRNA was het meest uitgesproken, vooral in de longen en de lever. Interventies moeten dus bij voorkeur gericht zijn tegen meerdere cytokines en in dit model lijkt er in elk geval een ruime tijdsperiode na het primaire insult beschikbaar waarin behandeling kan plaatsvinden.

Eerder onderzoek in ons laboratorium heeft aangetoond dat TNF-α spiegels in het plasma verhoogd zijn in de eerste 24 uur na injectie van zymosan, gevolgd door een opvallende piek vanaf dag 8. Daarnaast hebben vervolgexperimenten met het model aangetoond dat interventies gericht tegen TNF-α de symptomen van MODS kunnen afzwakken, hetgeen laat zien dat TNF-α een prominente rol speelt in de pathofysiologie. Aanvullend bewijs voor deze hypothese wordt geleverd door experimenten met TNF knockout-muizen. De volledige afwezigheid van TNF resulteert in een significant lagere sterfte in het ZIGI model (hoofdstuk 4). Daarnaast hebben TNF knockout-muizen op dag 22 een hogere gemiddelde lichaamstemperatuur en een hoger gemiddeld lichaamsgewicht dan de muizen van het wildtype. Overlevende dieren van beide groepen vertonen dan echter aanzienlijke en vergelijkbare microscopische schade in longen, lever en milt en een verhoogd orgaangewicht. Hoewel TNF-deficiënte muizen dus een significante verbetering laten zien in morbiditeit en mortaliteit, beschermt de afwezigheid van TNF blijkbaar niet volledig tegen de symptomen van MODS die door zymosan opgewekt worden.

Aangezien strategieën gericht tegen de werking van alleen TNF-α tot nu toe slechts ten dele succesvol gebleken zijn in het voorkomen van MODS in het muizenmodel, lijkt het aannemelijk dat er meer winst te boeken valt bij interventies die gericht zijn tegen meerdere cytokines tegelijkertijd.

Pentoxifylline (PTX) is een methylxanthinederivaat dat gebruikt wordt bij de

behandeling van chronische occlusieve arteriële aandoeningen vanwege zijn reologische werking. Daarnaast heeft PTX effecten op de ontstekingsreactie. Er is aangetoond dat het TNF-α, IL-1β en IL-6 spiegels in sepsismodellen kan verlagen. Het is bekend dat PTX fosfodiësterase remt, waardoor cyclisch adenosine monofosfaatspiegels in het cytoplasma verhoogd worden. Dit laatste remt sterk de genexpressie van TNF-α. Omdat PTX meerdere proinflammatoire cytokines tegelijkertijd kan tegenwerken is het mogelijk een geschikt middel om de gegeneraliseerde ontstekingsreactie die tot orgaanfalen leidt af te zwakken. We hebben een interventiestudie uitgevoerd waarin de effecten van PTX toediening onderzocht werden (hoofdstuk 5). *Ex vivo* remt PTX de door LPS geïnduceerde productie van TNF-α en IL-6 (maar niet van IL-1β) in J774.A1 macrofagen op een dosisafhankelijke manier, zowel op mRNA als op eiwitniveau. Wanneer zymosan-behandelde C57BL/6 muizen PTX toegediend krijgen, resulteert dit niet in een significant verbeterde overleving, lichaamstemperatuur, lichaamsgewicht of longscore. Ook zijn er geen significante verschillen in orgaangewichten gevonden tussen muizen die PTX kregen en muizen die PBS kregen. De TNF-α en IL-6 mRNA expressie in de lever is vergelijkbaar in beide groepen en dit is waarschijnlijk de belangrijkste oorzaak voor het uitblijven van positieve effecten op de morbiditeit en mortaliteit. Bij elkaar genomen bieden deze resultaten dus geen steun voor de hypothese dat PTX positieve effecten heeft bij de orgaanschade die door zymosan wordt opgewekt.

Tijdens ontstekingen en shock kunnen proinflammatoire cytokines en componenten van bacteriële celwanden het enzym nitric oxide synthase (iNOS) induceren. Het NO geproduceerd door iNOS wordt verondersteld weefselschade te veroorzaken, direct danwel indirect door de vorming van peroxynitriet. Wij hebben de rol van iNOS in het ontstaan van MODS bij muizen onderzocht door gebruik te maken van zowel een selectieve iNOS-remmer (aminoguanidine) als iNOS knockout-muizen (hoofdstuk 6). Kwantitatieve reverse transcriptase

polymerase chain reaction (RT-PCR) en immunohistochemie laten een sterk verhoogde expressie van iNOS messenger RNA (mRNA) en iNOS eiwit zien in de levers van C57BL/6 muizen in de derde – MODS-achtige- fase van het model. De *in vivo* toediening van aminoguanidine aan C57BL/6 muizen en de volledige afwezigheid van iNOS enzym (iNOS knockout-muizen) hebben echter geen van beide een gunstig effect op overleving, lichaamsgewicht en lichaamstemperatuur. Het long-, lever-, en miltgewicht en de longscores van de verschillende experimentele groepen zijn onderling vergelijkbaar. Deze resultaten pleiten sterk tegen een essentiële en oorzakelijke rol van iNOS bij het ontstaan van MODS bij muizen.

De extracellulaire matrix speelt een centrale rol in de structuur van weefsels en bij het instandhouden van homeostase. Het uiteenvallen van de weefselarchitectuur resulteert in orgaanfalen. Het remodelleren van de extracellulaire matrix, hetzij als onderdeel van de fysiologische homeostase en ontwikkeling hetzij als onderdeel van een pathologisch proces, gebeurt voor een groot deel door enzymen van de matrix metalloproteinase (MMP) familie, die bestaat uit een groeiend aantal gesecreteerde en membraangebonden metalloendopeptidasen. Bij ontstekingen spelen ze een belangrijke rol bij de interactie van cellen met hun omgeving. Bij diverse inflammatoire aandoeningen worden MMP's gedacht betrokken te zijn bij optredende weefselschade. MMP's worden ook in verband gebracht met systemische ontstekingen. Diverse onderzoeken suggereren dat MMP's een belangrijke rol spelen in het ontstaan van het adult respiratory distress syndrome (ARDS) en onlangs is aangetoond dat MMP-9 concentraties in de circulatie verhoogd zijn bij patiënten in kritieke toestand. Het is aannemelijk dat overactivatie van MMP's kan bijdragen aan de orgaanschade die bij MODS wordt waargenomen. De genexpressie van MMP's wordt gereguleerd door diverse factoren, waaronder cytokines. Er is een experiment gedaan waarbij de expressie en activiteit van verschillende gelatinases en collagenases werd bepaald in de lever, long, milt en nieren tijdens

MODS opgewekt door zymosan (hoofdstuk 7). De expressie van MMP-2 mRNA in leverweefsel van zymosan-behandelde muizen is sterk opgereguleerd. Voor MMP-9 is dit het geval voor alle onderzochte organen. In de lever, de longen, maar met name in de milt van zymosan-behandelde muizen wordt een significante toename van de activiteit van zowel pro- als actief MMP-2 en –9 gezien naarmate de tijd vordert. De activiteit van MMP-1 en –13 is erg laag in alle lever- en longmonsters. In de milten van zymosan-behandelde muizen wordt echter een hoge activiteit van MMP-1 en –13 waargenomen. Specifieke immuunkleuring voor MMP-2 wordt gezien in de lever en de milt, maar niet in de longen en in de nieren van zymosan-behandelde dieren. Kleuring voor MMP-9 is aanwezig in lever-, long- en miltweefsel van zymosan-behandelde muizen. Voor beide MMP's lijkt de kleuring beperkt te blijven tot fagocyten. Deze resultaten suggereren dat MMP's, vooral MMP-9, een rol spelen in de pathogenese van MODS. Interventiestudies met MMP-remmers zouden aanvullend bewijs voor deze hypothese kunnen leveren.

Aangezien MODS niet door één enkele mediator veroorzaakt lijkt te worden, lijken interventies gericht tegen meerdere mediatoren tegelijkertijd de meest hoopgevende benadering voor de toekomst. Het vinden of ontwerpen van substanties die meerdere mediatoren op een specifieke manier kunnen beïnvloeden zal hierbij een belangrijk onderwerp zijn. Eerdere studies die gericht waren tegen meerdere mediatoren zijn uitgevoerd met niet-specifieke behandelingen, terwijl biologische werkingsmechanismen van de gebruikte substanties vaak niet geheel duidelijk zijn; dit maakt het trekken van harde conclusies moeilijk. Hopelijk zullen specifieke interventies in de nabije toekomst mogelijk zijn, zoals het remmen van meerdere proinflammatoire cytokines tegelijkertijd, zonder andere cellulaire processen te beïnvloeden. Dit soort studies zal ons begrip van de onderliggende pathofysiologie van MODS verbeteren en zal uiteindelijk leiden tot betere behandelmethoden bij patiënten met (het risico op) MODS.

DANKWOORD

Promoveren doe je allerminst alleen, en daarom is een woord van dank op zijn plaats aan iedereen die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift. Laat ik allereerst mijn promotor, professor Goris, en co-promotor, professor Van de Meer, bedanken. Professor Goris, u staat aan de basis van dit proefschrift. Als “grote initiator” van het ZIGI model bent u al vanaf de jaren ’80 betrokken bij al het onderzoek dat op de afdeling met dit model plaatsvindt. Deze onderzoeken vormden de basis van waaruit ik kon vertrekken toen ik aan mijn onderzoek begon. Als chirurg bood u mij ook een frisse kijk vanuit een klinisch oogpunt, daar waar ik alleen kon buigen op laboratorium- en boekenwijsheid. Bedankt voor de prettige samenwerking.
Professor Van der Meer, bedankt voor de adviezen op cytokinengebied. Het valt niet mee om met een vooropleiding als voedingskundige wegwijs te worden in de wondere wereld der cytokinen. De wekelijkse cytokinenbesprekingen hebben zeker bijgedragen aan meer begrip op dit gebied mijnerzijds.
Het hebben van leuke collega’s vind ik een van de belangrijkste aspecten die van invloed zijn op de arbeidsvreugde, en ik vind dat ik daarbij op het lab van de Heelkunde erg geboft heb. Ik heb het lab al vanaf het begin leren kennen als een gezellig mannenclubje met wie het ook buiten werktijd goed toeven is.
Thijs Hendriks, jij bent als mijn directe begeleider de belangrijkste persoon geweest bij het tot een goed resultaat brengen van mijn onderzoek. Jouw kritische doch pragmatische manier van denken gaf mij de sturing die ik nodig had en bij het schrijven van artikelen zag jouw Engelse taalgebruik er toch telkens professioneler uit dan mijn aanvankelijke tekst, waardoor de leesbaarheid (en de publiceerbaarheid!) van de manuscripten aanmerkelijk toenam. Mijn dank hiervoor.
Erwin Waas en Arjan Nooteboom, wij hebben op ons AIO-kamertje samen erg veel lol gehad en heel wat lief en leed gedeeld, vaak op tijdstippen waarop we ook hard aan onze promotie hadden kunnen werken. Echter ook daarbuiten hebben we veel tijd met elkaar doorgebracht. Erwin, vooral in de eerste jaren

van mijn onderzoek zijn we regelmatig de bioscoop, kroeg, of beide ingedoken. Ik heb je in de loop der tijd erg leren waarderen ondanks, of misschien wel dankzij, het feit dat we vaak anders tegen dingen aankijken. Arjan, jij weet balorigheid tot kunst te verheffen. Dat merkte ik voor het eerst op het congres in Muenchen (ik heb zelden zo gelachen), maar natuurlijk ook later, toen we samen de aap gingen uithangen in een bandje. Jongens, jullie zijn mijn vrienden geworden en ik hoop dat we onze vriendschap ook na onze promoties kunnen voortzetten (ik eis op zijn minst een jaarlijks in de winter terugkerend "doctorweekend"!).

Ben de Man en Roger Lomme, wat zou het lab zijn zonder jullie? De hulp die ik van jullie heb gekregen omvat zoveel verschillende dingen… Roger, vooral de laatste periode heb je me veel geholpen met werkzaamheden waar ik niet altijd tijd voor, zin in, of de mogelijkheid toe had. Ben, jij hebt me wegwijs gemaakt in het werken met muizen en je hebt me heel vaak geholpen bij de werkzaamheden op het dierenlab, die niet zelden in de weekenden plaatsvonden. Bedankt jongens, natuurlijk ook voor de gezellige koffiepauzes waarvoor jullie doorgaans stipt om 10 en 3 uur op de AIO-kamer kwamen aankloppen ("labwerk is een kwestie van goed plannen").

Ik heb ook veel hulp gekregen van mensen van andere afdelingen van het UMC St Radboud. Ongeveer tien jaar geleden luisterde ik, zittend in Wageningse collegebanken, naar de verhalen van een vriendelijke man met guitig samengeknepen oogjes, niet wetend dat het ooit nog tot een professionele samenwerking zou komen. Deze man, Albert Verhofstad, wil ik graag bedanken voor zijn bijdrage aan het beoordelen van microscopische kleuringen en zijn adviezen hieromtrent, maar natuurlijk ook voor zijn bijdrage aan mijn teksten. Ook wil ik Vincent en Anneke van het pathologielab bedanken voor hun praktische hulp bij de kleuringen en het nemen van histologische foto's. Ik heb ook kleuringen verricht op het lab van (inmiddels professor) Joost Schalkwijk, waar Ivonne van Vlijmen en Mieke Bergers mij op weg hielpen met de

kleuringen en Joost me regelmatig bijgestaan heeft bij het beoordelen van de resultaten. Hartelijk bedankt hiervoor.
Ik wil verder iedereen op het lab van de algemene interne geneeskunde bedanken, met name Anneke Hijmans voor het mij bijbrengen van de grondbeginselen van de PCR-techniek en Ineke Verschueren voor haar RIA- en ELISA-bepalingen. Ik ben ook heel veel dank verschuldigd aan Louis van der Locht van het CHL. Louis, de keren dat ik (en vele anderen) met PCR-problemen bij jou ben komen aankloppen zijn talrijk. Steeds nam je de tijd voor me, terwijl je natuurlijk ook druk was met je eigen werkzaamheden. Mensen zoals jij, die verder kijken dan de belangen van alleen hun eigen afdeling, maken het Radboud als collectief sterker. Ik waardeer deze werkhouding zeer.
Ik ben ook dank verschuldigd aan Geert Poelen en de andere medewerkers van de SPF unit op het dierenlab. Jullie zijn altijd erg behulpzaam geweest en deden nooit ergens moeilijk over, dat is helaas vaak anders. Bedankt hiervoor. Michel van der Jagt, *fuule gak*, bedankt voor je bijdrage aan het iNOS-artikel. Je was de meest kleurrijke student die stage heeft gelopen op het lab in de periode dat ik er werkzaam was. Ik heb je humor erg gewaardeerd, en daarnaast ben je natuurlijk ook gewoon een erg aardige kerel.
Monique Jansen, mijn "voorgangster in het ZIGI model", jij hebt me meer geholpen dan je zelf waarschijnlijk beseft. Ik heb voortgebouwd op jouw werk, en aangezien het makkelijker is op een rijdende trein te springen dan hem zelf aan te duwen, heb ik bij mijn onderzoek weinig opstartproblemen gehad. Ook was het bij het schrijven van artikelen fijn om de kunst een beetje bij jou af te kijken. Dankjewel.
Dan zijn er tot slot nog mensen uit mijn privéleven wier morele steun op indirecte wijze heeft bijgedragen aan de totstandkoming van dit proefschrift. Het zou misschien wel geschreven zijn, maar zonder hen zou er niets aan geweest zijn! Martin, als iedere boom die wij over het leven opgezet hebben een echte zou zijn, zou niemand zich nog druk hoeven maken over het tropisch

regenwoud. Mark, het blijft na al die jaren inspirerend om jou als vriend te hebben. *Never a dull moment*…Luce, al vanaf het begin was het gezellig samen, hulde aan de “mannenvrouwen”! “Johan de droomeconoom”, altijd in voor voor gezelligheid en een biertje. Dank ook aan Jan-Willem, Wouter, (en nogmaals Arjan) voor de geweldige muzikale uitspattingen op de maandagavonden en de gezelligheid daaromheen. Jolie, Wouter (No.2), Kata, Sanne en Judith, bedankt voor de leuke tijd aan de Azaleastraat. Judith (no.2), wie had verwacht dat een cursus proefdierkunde romantiek zou kunnen opwekken? Bedankt voor de fijne tijd.

Thuis ligt de basis. Papa, mama en Johan, wie zou ik zijn geworden zonder jullie? De invloed die jullie hebben gehad op mijn ontwikkeling kan nauwelijks overschat worden. Dank jullie wel!

Ik heb de belangrijkste persoon tot het laatst bewaard: Annelieke, ik had nooit gedacht dat jij zou bestaan. Sinds ik jou ken voel ik mij een ander mens. Een beter mens. Ik voel mij ontzettend dankbaar dat ik mijn leven met jou mag delen.

OVER DE AUTEUR

CURRICULUM VITAE

Thomas Volman werd in 1974 geboren in het Limburgse plaatsje Buchten en behaalde in 1992 zijn VWO diploma aan het College Sittard. Vanwege zijn interesse voor biologie en scheikunde ging hij vervolgens “Voeding en Gezondheid” studeren aan de Landbouwuniversiteit Wageningen. Bij de vakgroep Humane Voeding en de vakgroep Fysiologie van Mens en Dier werden afstudeervakken gedaan, en er werd stage gelopen bij de afdeling “Animal & Poultry Science” op de Universiteit van Guelph, Ontario, Canada. In 1998 studeerde hij af. In datzelfde jaar werkte hij enkele maanden als practicumassistent bij de vakgroep Fysiologie van Mens en Dier. Na korte tijd bij het ID-DLO in Lelystad gewerkt te hebben, werd hij in 1999 aangesteld als Assistent-in-opleiding op het researchlaboratorium Heelkunde van het UMC St. Radboud te Nijmegen. Daar deed hij onderzoek naar het multi-orgaan disfunctie syndroom. Deze werkzaamheden resulteerden in dit proefschrift.